# The Role of the 3'-5' Exonuclease Activity of Herpes Simplex Virus Type 1 in Translesion DNA Synthesis

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by

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**General Background**

The ability of living organisms to pass genetic information on to their offspring is essential for the propagation of a species and for the evolution and adaptation of a population. Each living organism carries within each of its cells a complete copy of the genetic code which provides the instructions for its development. In order to pass this information along to future generations, organisms must have a means of copying their genomes so that all the necessary genes are present in the offspring with little or no alteration.

Whenever a cell divides it duplicates its DNA so that each cell may receive one copy of all of the genetic material for the organism. In eukaryotes, the double stranded genome is directionally unwound into two single strands and each is used as a template for the production of the nascent strand. Each template consists of a series of nitrogenous bases known as adenine (A), cytosine (C), guanine (G), and thymine (T). When a new strand of DNA is built on the template strand, (A) will normally pair with (T) and (C) will normally pair with (G). By maintaining these relationships, known as Watson-Crick base pairs, the genome can be accurately copied over and over with very few errors.

There are many cellular proteins involved in the process of duplicating a genome. The principal enzyme responsible for adding nucleotides to a growing DNA strand is DNA dependent DNA polymerase. DNA polymerase (pol) adds free nucleotides to a free 3' end of a new DNA strand, preferentially incorporating correct nucleotides that properly base pair with the template strand. Some polymerase molecules have an associated 3' to 5' exonuclease (exo) activity. This domain of the molecule is capable of removing nucleotides from the 3' end of the growing chain (1).

**Fidelity**

Cells have multiple mechanisms for maintaining fidelity during replication. These systems that ensure accurate copying of the genome take place both during replication and after replication has been completed. In general, polymerase enzymes are very selective for correct nucleotides, misincorporating only one in $10^4$ to $10^6$ nucleotides (1). When a misincorporation does occur, many polymerases posses a proofreading function that can remove incorrect bases immediately and allow correction by the polymerase. These two systems constitute the fidelity control that occurs during replication.

Cells also have mechanisms for avoiding errors that are not immediately corrected. If a polymerase attempts to replicate through damaged DNA, it may partly dissociate from the primer terminus (2). This allows nonreplicative, error-prone polymerases such as those of the Y-superfamily (3), to perform translesion synthesis. Translesion DNA synthesis is often mutagenic but is a favorable alternative to abortive replication that will certainly cause cell death. These enzymes are weakly processive (3) and allow the replicative polymerase to resume replication once a correctly paired primer terminus has been established (2). Cells also have highly specific repair systems that correct mispairs and lesions, such as thymine dimers and uracil, post replication. These post-replication repair systems are important for correcting errors in DNA before they are passed on to daughter cells where they may become permanently mutagenic.

**Polymerization**

To duplicate DNA, the polymerase must interact with the primer terminus and deoxynucleoside triphosphates (dNTPs). The polymerase must be able to sufficiently

bind to all four dNTPs. Herpes Simplex Type 1 (HSV-1) DNA polymerase also interacts with a processivity factor called UL42 (4). This protein has the same processivity enhancing function of its counterparts, such as PCNA in eukaryotic cells or the β clamp in *E. Coli* (4). Unlike PCNA or β, however, UL42 binds both to the polymerase molecule and the DNA itself, and its DNA binding activity is necessary for stimulation of the polymerase processivity (4).

Polymerase domains consist of three subdomains called the fingers, palm, and thumb (5). The polymerase binds to the primer template through interactions in the palm. The fingers of the molecule rapidly open and close around the bound DNA. It is these fingers that deliver the dNTP to the polymerizing site. It has been suggested that a correctly paired dNTP brought to the polymerizing site will cause a shift in the conformational equilibrium that will favor the closed state thus keeping the dNTP in the site longer and facilitating polymerization (6).

**Exonuclease**

The associated exonuclease (exo) activity of the HSV polymerase, as well as many other polymerases, is responsible for proofreading the nascent strand synthesis and removing incorrect nucleotides as they are incorporated. The exo domain can act upon single stranded DNA or melted double stranded DNA substrates since it must bind single stranded DNA for activity (7). Therefore, in the context of a primer-template, the preferred substrate for exo activity is a mispair at the primer terminus, whereas a proper Watson-Crick basepair is preferred by the polymerization domain (7). The polymerase and exo activities are in constant competition for the primer terminus and equilibrium is established between the two domains (1). This equilibrium can be influenced by the

availability of dNTPs, primer template mispairs, distortions in the primer template, or the amount of dNMP in the system (7). Under optimal conditions for replication, and in the absence of misincorporation, polymerase activity predominates. Exo activity is not readily observed when the polymerase is acting on a matched primer terminus in the presence of all four nucleotides (1). It has also been shown that the equilibrium can be pushed toward polymerization even on damaged DNA templates by providing the polymerase with high levels of dNTPs (7).

**Contributions to Fidelity**

The polymerase and exo domains each contribute to the overall fidelity of the enzyme. The first step is the correct nucleotide selection by the polymerase. Polymerase has been found to have a 10-1000-fold higher affinity for the next correct nucleotide than for an incorrect one (5). It has been suggested that the binding of a correct nucleotide causes a conformational change in the dNTP binding subdomain of the polymerase which activates the polymerase for catalysis more so than an incorrect nucleotide (reviewed in 8). This preference for correct nucleotide selection accounts for most of the fidelity of a replicative polymerase. Once the fingers have brought the nucleotide into the catalytic site, the enzyme must overcome the free energy of a transition state to form the phosphodiester bond. Showalter and Tsai have proposed that the differences in free energy presented by correct versus incorrect nucleotides constitute the main fidelity checkpoint rather than the conformational change (8). Whatever the mechanism, polymerase molecules can discriminate between correct and incorrect nucleotides such that they achieve misincorporation rates from $10^{-3}$ to $<10^{-6}$ (5).

Most replicative polymerases also have either an intrinsic or an associated 3'-5' exo activity that is another means of avoiding errors (9). The exo performs a proofreading function which removes misincorporated nucleotides from the 3' end of a growing chain. Distortions in the physical structure of the primer terminus, caused by misincorporated nucleotides or a damaged DNA template, cause the primer terminus to be translocated to the exo domain (10). Excision by the exo domain gives the polymerase another chance to incorporate the correct nucleotide. In the presence of noncoding template lesions, such as abasic sites, the primer terminus will be translocated to the exo site and back to the polymerase domain many times in futile cycles of incorporation and excision (7). It has been suggested that this process may be important for signaling cell cycle control proteins in response to damaged DNA (7) as well as stalling to allow Y family translesion synthesis polymerases to come in and copy across the lesion (2). The presense of an active exo domain has been shown to improve fidelity of replication of undamaged templates six-fold to eight-fold over polymerase alone (7).

The overall fidelity of DNA replication depends on contributions from the polymerase, the exo, as well as accessory proteins. In addition, post replication repair and translesion synthesis play vital roles in correcting replication mistakes.

**DNA Damage**

As a cell interacts with its environment, its genome can be damaged and genetic information can be lost or changed. This damage can be caused by free radicals, chemicals, or by forms of radiation such as gamma rays, x-rays, and ultraviolet light (9). Errors can also occur as part of the normal physiology of the cell. The base cytosine spontaneously undergoes deamination and becomes deoxyuridine, a base not normally

found in DNA (11). Deoxyuridine pairs with deoxyadenine thus causing a C→T transition mutation in the next round of replication. DNA can also be damaged during replication. Polymerases can incorporate an incorrect base which causes a change in the sequence of that DNA strand.

One of the most common types of damage observed in DNA is an apurinic/apyrimidinic site, otherwise known as an AP or abasic site (12). These are lesions on DNA where the nitrogenous base has been cleaved at the N-glycosidic bond but the phosphate backbone of the DNA has been left intact (13). These lesions occur frequently in eukaryotes as part of a repair pathway that removes uracil from DNA. Normally these residues are removed prior to DNA replication. Since AP sites have no base, they provide no coding information to the nascent strand. Thus, if a polymerase incorporates a random nucleotide opposite an abasic site, there is a 75% chance there will be insertion of an incorrect base. However, the chance of mutation is increased by the fact that many polymerases follow the "A-rule," whereby they preferentially incorporate deoxyadenosine across from an abasic site, which will result in a transition mutation (2).

Damage to DNA has many consequences for cells. Changes in the coding sequence due to misincorporation of nucleotides or due to a lack of information from an abasic site can diminish or destroy gene function. Damage outside the gene coding region can affect promoters and enhancers and therefore affect multiple genes. Changes to genes critical for cell function may cause disease or death for the organism that incurs the damage. Clearly, accumulation of mutations increases the probability for lethal hits.

## Herpes Simplex Virus Type 1

Herpes simplex virus type 1 (HSV-1) uses the same basic mechanisms of DNA replication as model systems such as *E. coli*. HSV-1 encodes an origin-binding protein called UL9 that is analogous to DnaA in *E. coli* and is thought to be responsible for unwinding duplex DNA at the origin of replication (4). The helicase/primase activity is accomplished by a heterotrimeric complex composed of UL5, UL52, and UL8 (4). The product of the UL29 gene is a single stranded binding protein known as ICP8. This molecule stabilizes single stranded DNA as it is unwound and allows the polymerase molecules access to the templates (4).

HSV-1 also encodes its own DNA polymerase. UL30 encodes the catalytic subunit which possesses both a polymerase domain and an associated exo domain (4). HSV-1 polymerase has been shown to incorporate an incorrect nucleotide once every 300 bases in pre-steady state kinetic experiments (1). This is a high error rate that, without the editing function of the exo domain, could lead to many mutations within the genome. As mentioned before, HSV-1 polymerase binds to a processivity factor called UL42 and forms the polymerase holoenzyme (1). UL42 has been shown to bind to DNA and polymerase (1) and is necessary for viral replication (4).

## HSV Pathogenesis

Nine of the over 130 species of the family *Herpesviridae* have been shown to infect human beings, including HSV-1 (1). HSV-1 commonly causes oral and genital infections, and can cause ocular infections which can lead to blindness. HSV-1 is so prevalent that is has been estimated that 85% of the adult population worldwide has antibodies against HSV-1 (14).

HSV-1 is transmitted mostly through nongenital contact with infected secretions (15). HSV is capable of causing a latent infection in an individual following an acute infection. The virus remains dormant in nerve cells that innervate the infected area until it is induced into a lytic cycle by stress, sunlight, emotional stress, fever, and immunosupression (14). In this way, the virus avoids the human immune system and the host is infected for life, with symptomatic outbreaks occurring intermittently.

There is no known cure or effective vaccine for HSV. Nucleoside analogs such as acyclovir have been in wide use to control the infection. Acyclovir is a nucleoside analog that is added to a growing DNA strain only when it is phosphoylated by the viral thymidine kinase and converted to a triphosphate by cellular enzymes. This drug terminates the growing DNA chain and thus prevents viral DNA replication. However, resistant strains of the virus are present in 5% of immunocomprimised patients (1).

Several new drugs have been developed to treat patients with Acyclovir-resistant infections. Drugs such as Foscarnet and Vidarabine act by inhibiting the viral polymerase (15). Others, such as Trifluorothymidine and Cidofovir act as nucleoside analogs that can be incorporated in the absence of active viral thymidine kinase activity (15).

Drug-resistant virus is a result of the dynamic structure of a virus population. In any given population, there exist many mutants either deficient or exceedingly proficient in certain metabolic processes. These mutants are often resistant to drugs which target processes performed by the majority of the virus population. Mutations of this type are selected, even if rare, to survive the drug treatment and propagate to form a drug-resistant infection.

Polymerase fidelity is extremely important in the formation of drug-resistant mutants. Unfaithful copying of the viral genome may cause the mutations that allow the virus to survive the drug treatment. In this way errors made while copying the DNA may prove beneficial for the virus population at large. The rise of drug resistant strains of viruses has proven the need for drugs that block several metabolic pathways including viral DNA replication. The more that is discovered about the mechanisms of HSV replication, the better these drugs will be able to target the infection. Additionally, the insight gained through the study of herpesvirus may lead to breakthroughs in control of other important families of viruses such as poxvirus and HIV.

Effective treatments and vaccines for a wide range of viruses have become even more important as the threat of bioterrorism continues to grow. Viruses such as smallpox and viruses which can cause hemorrhagic fevers (e.g. Ebola and Marburg viruses) are considered especially dangerous as biological weapons (16). Since smallpox and HSV are DNA viruses it seems reasonable that investigating HSV as a model for replication of DNA viruses may help find effective drugs to combat smallpox as well.

**Recent Research**

Recent work in the field of replication fidelity and AP site bypass has provided important insight into the role of the 3' to 5' exo activity in maintaining fidelity. It has been shown that when a DNA polymerase is faced with a non-instructional lesion, it will preferentially incorporate an A residue into the growing chain opposite the lesion (17). This preference had been termed the "A-rule." The preference for adenine is highly mutagenic since so few AP sites are likely to be formed from thymine precursors.

Comparisons of exo-proficient and exo-deficient polymerase in a T4 system has shown that those deficient in proofreading activity are better able to replicate through an AP site (12). This observation, along with the fact that all of the known Y family translesion synthesis polymerases lack a proofreading function, is further evidence that the exo domain is important for inhibiting translesion synthesis and preventing mutagenesis (7).

A steady-state kinetic study of the ability of the HSV-1 polymerase to incorporate nucleotides opposite an abasic site has shown that incorporation efficiency for exo deficient polymerase is on the same order of magnitude as that of wild-type enzyme (1). This study also showed that the ability of the polymerase to extend the primer by one nucleotide was severely inhibited for both wild-type and exo-deficient polymerase. The wild-type enzyme was impeded to a much greater degree than the mutant polymerase, suggesting that the exo activity plays a significant role in recognizing the AP site and preventing extension (1).

Work done in the lab of Dr. Deborah S. Parris has shown that HSV-1 polymerase proficient in exonuclease activity can engage in idling-turnover reactions where the dNTP across from the AP site is successively incorporated and excised many times, causing a pause in the replication process (18), and that the rate of this turnover reaction is increased by the processivity factor, UL42 (19). It has also been shown that presence of UL42 does not increase translesion synthes (1). Thus, UL42 has been implicated in preventing dissociation of the polymerase from the primer terminus and thus allowing more time for the excision of misincorporated bases.

**Current Research**

These findings have raised many intriguing questions concerning the nature of the interaction between the primer terminus and the polymerase that allow the polymerase to sense the lesion and attempt to correct it. Specifically, can the polymerase sense the damaged DNA once it has already replicated past it and go back to fix it? My work has attempted to characterize the interaction of the polymerase and primer terminus once the polymerase has replicated several bases beyond the AP site.

The hypothesis governing this study is that the AP site will remain a barrier to extension several bases beyond the site of damage, and that the amount of interference will decrease with each base past the AP site. My studies have been designed to achieve two specific aims:

- To show that the exo activity is important for preventing extension through an AP site
- To show that the exo domain is capable of detecting an AP site following incorporation of several correct nucleotides downstream of the lesion

## Materials and Methods

**Cells and Viruses:** *Spodoptera frugipera* 9 (Sf9) cells were propagated at 27°C in TNM-FH insect medium containing 100 units of penicillin per ml, 100 µg of streptomycin sulfate per ml, and 10% heat-inactivated fetal bovine serum. The cells were recultured every five days at a 1:10 ratio. Recombinant baculovirus expressing HSV-1 wild-type pol was a generous gift of Dr. Robert Lehman (Stanford University, Palo Alto, CA). The recombinant baculovirus expressing the exo-deficient (D368A) pol was a kind gift of Dr. Charles Knopf (Heidelberg, Germany). Virus stocks were prepared by infecting Sf9 cells at a multiplicity of infection (MOI) of 0.5 plaque forming units (PFU) per cell and harvesting the supernatant 7 days post-infection (p.i.).

**Protein Purification:** Sf9 cells were infected (MOI of 5 PFU/cell) with recombinant baculovirus expressing HSV-1 wild-type or D368A exo-deficient (exo$^-$) polymerase as described previously (1), and cells were harvested 40 hours p.i. Tubes containing infected Sf9 cells were centrifuged at 175xg for 10 minutes in a Beckman TJ-6 centrifuge, washed in cold Tris-buffered saline (TBS), centrifuged for another 10 minutes at 175xg, and resuspended in hypotonic buffer (RSB) for 10 minutes on ice. EDTA was added to achieve a final concentration of 5 mM. An equal volume of 2X lysis buffer (see Table 1) was then added. The extract was gently shaken at 4°C for 30 minutes then centrifuged for 30 minutes at 71,000xg in a Beckman L-70 ultracentrifuge, and the supernate was collected. Protein fractionation was conducted at room temperature using a Fast Protein Liquid Chromotography system (Amersham Biosciences, Piscataway, NJ), and fractions were collected on ice. Whole cellular extracts were dialyzed in B2 buffer and clarified by centrifugation at 70,000 xg. The extract was loaded onto a 15 ml DEAE-Sepharose ion-exchange column (Amersham Biosciences,

Piscataway, NJ) equilibriated with B2 buffer. Bound proteins were eluted with a 60 ml linear salt gradient of 0-0.5 M KCl in B2 buffer. Peak fractions containing the desired protein were loaded onto a 5 ml Hydroxyapatite type II column (Biorad, Hercules, CA) equilibriated with buffer D. The presence of pol protein and enzymatic activity in column fractions was determined by immunoblotting with pol-specific antibody, and the ability of each fraction to incorporate nucleotides on an activated calf thymus DNA template as previously described (19). The pol-containing fractions were collected and dialyzed in buffer C and loaded onto a 10 ml Heparin-Sepharose column. Fractions were collected over a 60 ml linear salt gradient from 0-1 M KCl in buffer C. Pol-contaning fractions were pooled and diluted with buffer C and loaded onto a 10 ml phosphate cellulse column (Whatman, Clifton, NJ) that was equilibriated with buffer C. Proteins were eluted over a 60 ml linear gradient from 0-1 M KCl, pooled, and concentrated by ultracentrifugation using Centricon-100 ultrafiltration units (Amicon, Bedford, MA).

**Nucleoside Triphosphates and Synthetic DNA:** dNTPs were purchased from Amersham Biosciences. [$\gamma$-$^{32}$P] ATP was purchased from MP Biomedicals, LLC. Activated calf thymus DNA was purchased from Sigma. Synthetic DNA primers and templates were obtained as PAGE-purified products from Integrated DNA Technologies, Inc. (Coralville, IA) A 1',2'-dideoxyribose (dSpacer) was used to mimic a stable AP site in some 67 mer templates.

**Preparation of Synthetic Primer-templates:** The primer strands used were of lengths varying between 47 and 49 nucleotides containing identical sequence in the first 47 residues and complementary to the 3' end of the 67 mer template strand (Table 2). The primers were radioactively labeled at the 5' end using [$\gamma$-$^{32}$P] ATP and T4 polynucleotide kinase. Unincoroprated [$\gamma$-$^{32}$P] ATP was removed from DNA by

ProbeQuant™ G-50 Micro Columns. Primer-template (P/T) partially duplex DNA was created by heating the primer with 20% excess template in 20mM Tris-HCl, pH 7.5, and 100 mM KCl to 57°C for 3 minutes followed by slow cooling to room temperature. Annealed primer-templates were stored at 4°C.

**Steady-state Extension Assay:** Polymerase extension assays were carried out in buffer containing 10 mM Tris-HCl pH 7.4, 1 mM dithiothreitol (DTT), 1 mM EDTA, 400 µg/ml bovine serum albumin (BSA), and 50 mM KCl. Enzyme (25 nM) was preincubated with 250 nM P/T. The reaction was initiated by adding an equal volume of buffer containing $MgCl_2$ (6 mM). The final enzyme and P/T concentrations were 12.5 nM and 125 nM, respectively. The reaction was incubated in a 37°C water bath and stopped by the addition of excess 0.3 M EDTA. Reaction products were separated by denaturing polyacrylamide gel electrophoresis (PAGE) using 12% acrylamide gels containing 7 M urea. Gels were exposed to a storage phosphor screen and the radioactivity in the sequence indicated was quantified using a Typhoon 9200 Variable Mode Imager (GE Healthcare, formerly Amersham Biosciences) and ImageQuant v5.2. Radioactivity in a given band was normalized to the total radioactivity in the lane to minimize loading error. The amount of fully extended product was plotted as a function of time and fit to either a linear or hyperbolic curve as indicated. Regression analysis of the extension products was performed using SigmaPlot v9.0. Data from nucleotide concentration assays was fit to the Michaelis-Menten function as shown by the following equation; where $V_0$ is the observed rate of extension, $V_{max}$ is the maximum rate of extension, and $K_m$ is the concentration of dNTP at half $V_{max}$.

$$V_0 = \frac{V_{\max}[\text{dNTP}]}{K_m + [\text{dNTP}]} \qquad \text{(Eq. 1)}$$

## Results

Determination of the role of the exo activity of the HSV-1 pol required the purification and use of an exonuclease-deficient pol (D368A) as previously described by Song et al, (2003). D368A pol was expressed in Sf9 cells by recombinant baculovirus and purified as described in Materials and Methods. Proteins in fractions that contained pol activity were separated by SDS-PAGE and visualized by staining with Coomassie Blue to determine purity (Fig 1).

### AP site causes barrier to extension

Previous results from mismatch extension experiments have shown that the efficiency for extension of a forced mismatch is decreased relative to a properly paired primer terminus (1). Furthermore, it was shown that this impediment to extension is enhanced by the presence of an active exo domain (1). Kinetic experiments performed with AP site-containing templates showed that exo-deficient HSV polymerase (pol) was more efficient than WT pol at extending primers on an AP-damaged template, even though both enzymes were similarly efficient at nucleotide insertion opposite an AP site (1).

To determine whether a damaged template acts as a barrier to extension after the polymerase has replicated past it, a primer/template construct was created such that the primer terminus was one nucleotide downstream of the AP site. The experimental setup is shown schematically in Figure 2 This will be referred to as the (+1) primer. It has been shown that polymerases, including HSV-1 pol, will preferentially insert an (A) residue across from abasic lesions (2). For this reason, this primer, and all others used in this study, contained an (A) residue opposite the AP site on the template. WT pol (12.5 nM)

was preincubated with the (+1) primer/template (P/T) (125 nM) in the presence of EDTA as described in Materials and Methods. The reactions were performed with excess dNTP (1 mM) to ensure that this reagent would not be limiting. Extension reactions were initiated by addition of $MgCl_2$, and samples were removed from a bulk reaction at various times and injected into tubes containing 0.3 M EDTA to stop the reaction. The samples were separated by denaturing gel eletrophoresis, and extension products were quantified and plotted as a function of time. Steady-state conditions were determined to be present during the times when the plot remained in the linear range. Some reactions never achieved or approached a maximum amount of extension product and displayed linear kinetics over the entire timecourse (Fig. 3b). However, in other reactions, steady –state conditions existed for only a short period, after which time the P/T became limiting (Fig 4b).

Extension reactions performed with WT enzyme on the (+1) primer show that the amount of unextended, undamaged primer/template (67T) construct became limiting after 100s (Fig 4), in which case the data fit well to the Michaelis-Menten equation (Eq. 1). By contrast, poor extension of the AP site-containing primer/template (67SP) construct by the WT pol permitted steady-state conditions throughout the 600s period of the experiment (Fig. 3). These results suggest that the AP site is preventing extension of the primer when the site of damage is 5' to the primer terminus.

Figure 3a shows that the WT pol created excision products (<47nt) while trying to extend a primer on a damaged template. These excision products were not observed in the experiment performed with the undamaged template (Fig. 4a). The presence of these excision products demonstrates that the exo activity is more prevalent in the presence of a

damaged template. Although the amount of available unextended primer appears to become limiting at 360s, the reaction maintained linear kinetics due to the presence of exo products, which were quantified as part of the total activity in the lane.

**Exo Activity Reinforces Lesion**

In the presence of an AP site, the WT enzyme created many excision products smaller than the original primer, while few if any excision products were ever observed with the undamaged template (compare Fig. 3a with Fig. 4a) . These results suggest that the exo activity plays a role in preventing extension when an AP site is present. It was hypothesized that the presence of exo activity would reinforce the lesion and would prevent extension to a greater degree than if no exo activity was present. To test this hypothesis, a mutant HSV-1 pol subunit that lacks exo activity was employed. D368A pol contains a point mutation in the conserved exo site I domain of HSV-1 pol. This mutant pol has been shown to lack all exo activity while retaining full polymerization activity (18). We compared efficiencies of WT and exo deficient ($exo^-$) pols for extension to test the role of the exo activity in preventing primer extension on damaged templates. Steady-state conditions were determined by running kinetic experiments with the $exo^-$ pol in the context of the (+1) primer, as described above. With the $exo^-$ pol, the 67T P/T became limiting by 60s (Fig. 5b) and the 67SP (AP containing) P/T became limiting by 300s (Fig. 6b). Neither showed any excision products throughout the timecourse (Figs. 5a,6a). These results suggest that the AP site remains a barrier to extension even in the absence of exo activity, albeit to a lesser extent than for the WT pol. The products extended to less than full length (<67) observed in each experiment suggest that the enzyme partially extends the primer, but dissociates before full extension

is achieved. Therefore, the enzyme is much more likely to bind to an unextended P/T since it is in excess. This process of dissociation and reassociation to an unextended primer results in populations of partially extended P/Ts. The strong band observed near the 59nt position is likely due to secondary structure in the template that is causing the enzymes to dissociate more frequently at that position. It was observed in essentially all of experiments.

To determine the efficiency of extension of the (+1) primer by the WT enzyme, reactions were performed for a constant time period with varied concentrations of dNTP (0-1000 μM). The time for terminating these reactions was chosen from the linear range of the timecourse for the respective template. By ensuring steady-state conditions in this way, it was possible to measure the apparent maximum rate of extension ($V_{max}$) and equilibrium binding constant ($K_m$) for the enzyme and to calculate the efficiency ($V_{max}/K_m$) of extension using Equation 1. The reaction of the WT pol was allowed to proceed for 60s on the undamaged template and for 300s on the AP site-containing template. Experiments in which the WT pol was incubated with the damaged template showed an extremely low, unquantifiable amount of extension at all concentrations of dNTP (Fig. 7), while extension of the undamaged P/T proceeded with an efficiency of $1.6x10^{-2}$ $sec^{-1}$ (Fig. 8). These results demonstrate that an AP site one nucleotide upstream of the primer terminus constitutes a severe barrier to extension.

To determine the role of the exo domain in reinforcing this barrier, the efficiencies of extension for the exo-deficient pol on each template was determined using various nucleotide concentrations. The reactions were allowed to proceed for 30s for the $exo^-$ pol on both templates (Figs. 9, 10). On the undamaged template, the $exo^-$ pol

extended with an efficiency of $3.5x10^{-1}$ $sec^{-1}$, whereas the efficiency was $3.8x10^{-2}$ $sec^{-1}$ on the damaged template, a 10-fold reduction (Results summarized in Table 4). These results reinforce the hypothesis that the AP lesion on the template is a barrier to extension and is sensed even in the absence of exo activity. However, the $exo^-$ pol also showed a much greater ability to extend the damaged P/T than did the WT pol (See Table 3). Taken together, these results suggest that although the exo activity is not essential for the ability of the pol to sense a damaged template, it plays a major role in preventing extension.

**AP site is sensed at least 3 nucleotides upstream of the primer terminus.**

To determine whether the AP lesion could be sensed by the pol further from the primer terminus than one nucleotide downstream, primers that continued 2 and 3 bases past the AP site (+2 and +3) were tested for extension. Steady-state conditions were determined as described previously, and the results are summarized in Table 3. Nucleotide concentration dependence assays were performed, as described, to determine the efficiency of extension of each enzyme and template pair for the (+2) and (+3) primers (Table 3). The WT pol exhibited very little extension at any concentration of nucleotides for the (+2) primer on the damaged template, and created many excision products (Fig. 11). The efficiency for extension was $2.6x10^{-5}$ for the damaged template, a 300-fold decrease from the efficiency of the undamaged template ($8.8x10^{-3}$) (Fig. 12). Extension efficiencies on the (+3) primer were not determined. However, kinetic experiments performed with WT pol on a (+3) P/T construct showed a greater rate of extension and no excision products (Fig. 13), whereas a slower extension rate and more excision products were observed with the damaged template (Fig. 14). The extension

efficiency of the exo$^-$ pol on the undamaged template showed no greater than a 5-fold difference on any of the three primers used (Table 4). Likewise, on the damaged template, the difference in extension efficiency was no greater than 3.5-fold. However, the efficiency of extension was lower for every primer on the damaged template than for any primer on the normal template. These results suggest that the AP site was sensed by the pol subunit even in the absence of exo activity, and even when removed from the primer terminus by up to 3 nucleotides.

By comparing the two enzymes, the WT pol showed a severely reduced efficiency of extension of an AP site-containing P/T even at the (+3) position, while the exo deficient pol showed less of a reduction in efficiency. This suggests that the presence of exo activity reinforces an AP lesion and prevents significant extension.

**Discussion**

Since AP sites are one of the most common forms of damage to DNA, their detection and subsequent repair is an integral part of maintaining DNA fidelity. The data presented here show that the HSV-1 pol can detect an AP site even after it has replicated up to three bases beyond the damaged site. This result suggests that there is some difference between the primer-terminus of an undamaged DNA duplex and one containing an AP site, and that this difference prevents the pol from extending damaged DNA. It has been shown that the pol subunit will readily insert nucleotides across from an AP site (1). New data presented here demonstrates that the fidelity checkpoint in translesion syntheses may include a backward-looking mechanism that acts more by preventing extension, than by preventing insertion.

Importantly, the prevention of extension past an AP site takes place with or without an active exo domain, demonstrating that the pol subunit is sufficient for detection of the AP site. Extension efficiencies for the exo-deficient pol were lower on damaged templates than on undamaged ones, showing that even without exo activity, the AP site presents a barrier to extension. This result draws an important distinction between the act of detecting the lesion, and reinforcing it. In every case, the WT pol showed a lower efficiency of extension on a damaged template than did the exo$^-$ pol, demonstrating that the exo activity, although not necessary for detection, plays a role in enhancing the barrier to extension.

Indeed, the exo activity probably plays more than one role in preventing extension of damaged DNA. In the experiments described here, the primer terminus annealed to the damaged template may have greater potential for melting due to the strain put on the primer strand by the conformation of the A:AP duplex. This process would create a region of ssDNA at the primer terminus that would be a preferred substrate for the exo domain. Thus, if the pol domain senses the lesion and switches the primer terminus to the exo domain, there will be a very high chance of excision. The other possible role for the exo domain is to cause idling turnover reactions at the AP site and thus stall the replication fork. It has been shown that WT pol can successively incorporate and excise mismatched nucleotides by switching back and forth between the pol and exo domains (1). In the case of an AP site, there is no correct nucleotide that can be added, so the enzyme may try to incorporate many nucleotides and excise them in a series of futile cycles without extending the primer (Fig. 21).

The accumulation of excision products smaller than the original template that are created by the WT pol on a damaged template suggest that the exo activity may predominate as the initial activity of the enzyme when it binds to a primer terminus. If the pol activity predominated, then one would expect to see few excision products since the primer would be immediately extended up to the AP site, where it would encounter the extension barrier. However, if the exo domain was the first to act on the primer terminus following association, then the successive rounds of binding and dissociation by the enzyme during steady-state conditions could result in the observed excision products.

Crystallography studies have shown that some polymerases can detect mispaired DNA up to 4 base pairs in from the primer terminus, and trigger an exo activity to attempt to correct the mistake (20). Although the crystal structure of HSV-1 pol has not been solved, the data presented here demonstrate this same kind of backward-looking error detection. This ability is a fail-safe mechanism to avoid extension of the many AP sites that are likely to accumulate during HSV replication. Further studies of the role of the exo activity of HSV-1 pol in translesion synthesis should include a characterization of the effect of the processivity factor (UL42) of the polymerase on primer extension, as well as further studies on the distance dependent effects of the kinetic barrier.

| Buffer | Mix |
|---|---|
| RSB | 10mM Tric-HCL, pH 7.4, 10mM NaCl, 3 mM $MgCl_2$ |
| 2x Lysis | 20mM Tris-HCl, pH8.2, 2 M NaCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride (PMSF) |
| B2 | 20 mM Tris-HCl, pH 8.2, 50 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol |
| C | 20 mM Hepes, pH 7.6, 50 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol |
| D | 10 mM $Na_2HPO_4$, pH 7.0, 100 mM NaCl, 5 mM 2-mercaptoethanol |

**Table 1 – Buffers Used in Protein Purification**

| Primer/template | Sequence |
|---|---|
| **47C/67N (+1)** | 5′-GCCACTACGACACCTTGATCGCCTCGCAGCCGTCCAACCAACTCAAC-3′<br>3′-CGGTGATGCTGTGGAACTAGCGGAGCGTCGGCAGGTTGGTTGAGTNGGTCTTGTTTCCAGTCGTTTA-5′ |
| **48C/67N (+2)** | 5′-GCCACTACGACACCTTGATCGCCTCGCAGCCGTCCAACCAACTCAACC-3′<br>3′-CGGTGATGCTGTGGAACTAGCGGAGCGTCGGCAGGTTGGTTGAGTNGGTCTTGTTTCCAGTCGTTTA-5′ |
| **49A/67N (+3)** | 5′-GCCACTACGACACCTTGATCGCCTCGCAGCCGTCCAACCAACTCAACCA-3′<br>3′-CGGTGATGCTGTGGAACTAGCGGAGCGTCGGCAGGTTGGTTGAGTNGGTCTTGTTTCCAGTCGTTTA-5′ |

**Table 2 – Sequences of Primers and Templates Used**

| P/T | Enzyme | Fig. | Max Time for Steady-State (s) | Nucleotide Concentration Dependence Reaction Time (s) |
|---|---|---|---|---|
| **48/67T** | **WT** | **15** | 120 | 60 |
| | **D368A** | **16** | 120 | 15 |
| **48/67SP** | **WT** | **17** | NL | 300 |
| | **D368A** | **18** | 120 | 15 |
| **49/67T** | **WT** | **13** | NL | I |
| | **D368A** | **19** | 180 | 30 |
| **49/67SP** | **WT** | **14** | NL | I |
| | **D368A** | **20** | 300 | 30 |

**Table 3 – Determination of Steady-state conditions from Kinetic Experiments**

| | Template | Primer | $V_{max}$ (μM/s) | $K_m$ (μM) | Efficiency ($sec^{-1}$) |
|---|---|---|---|---|---|
| **D368A** | **67T** | **47** | 1.40 | 3.97 | $3.5x10^{-1}$ |
| | | **48** | 2.81 | 15.61 | $1.8x10^{-1}$ |
| | | **49** | $5.92x10^{-1}$ | 8.11 | $7.3x10^{-2}$ |
| | **67SP** | **47** | $6.53x10^{-1}$ | 17.01 | $3.8x10^{-2}$ |
| | | **48** | 2.53 | 180.35 | $1.4x10^{-2}$ |
| | | **49** | $4.37x10^{-1}$ | 8.88 | $4.8x10^{-2}$ |

| | Template | Primer | $V_{max}$ (μM/s) | $K_m$ (μM) | Efficiency ($sec^{-1}$) |
|---|---|---|---|---|---|
| **WT** | **67T** | **47** | $8.72x10^{-1}$ | 53.6 | $1.6x10^{-2}$ |
| | | **48** | $7.42x10^{-2}$ | 8.35 | $8.8x10^{-3}$ |
| | | **49** | I | I I | |
| | **67SP** | **47** | NE | NE | NE |
| | | **48** | $8.64x10^{-2}$ | 3225.17 | $2.7x10^{-5}$ |
| | | **49** | I | I I | |

**Table 4 – Summary of apparent $V_{max}$, $K_m$, and Efficiency calculations for all P/T:Enzyme pairs. Abbreviations: NE, negligible extension; I, indeterminate.**

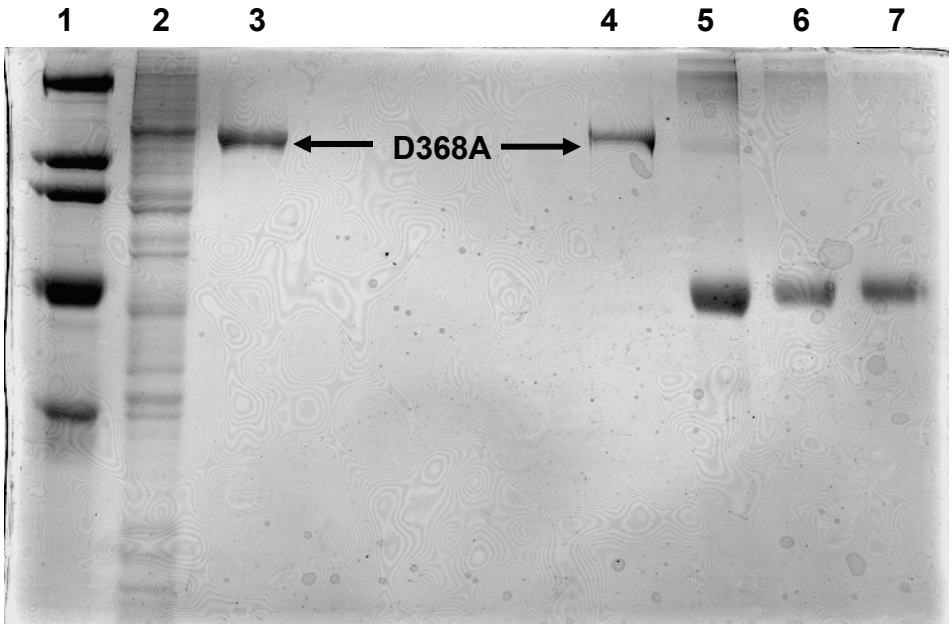



**Fig. 1**

**Figure 1:** Purification of D368A exonuclease deficient polymerase. Enzyme was harvested from Sf9 cells infected with recombinant bacculovirus and purified by FPLC. Proteins were separated by electrophoresis through SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, protein size marker. Lane 2, eluent from DEAE column (10 µg). Lane 3, pure D368A (2 µg). Lane 4, second pure D368A (2 µg). Lanes 5-7, BSA protein concentration standards, 4 µg, 2 µg, and 1 µg respectively.

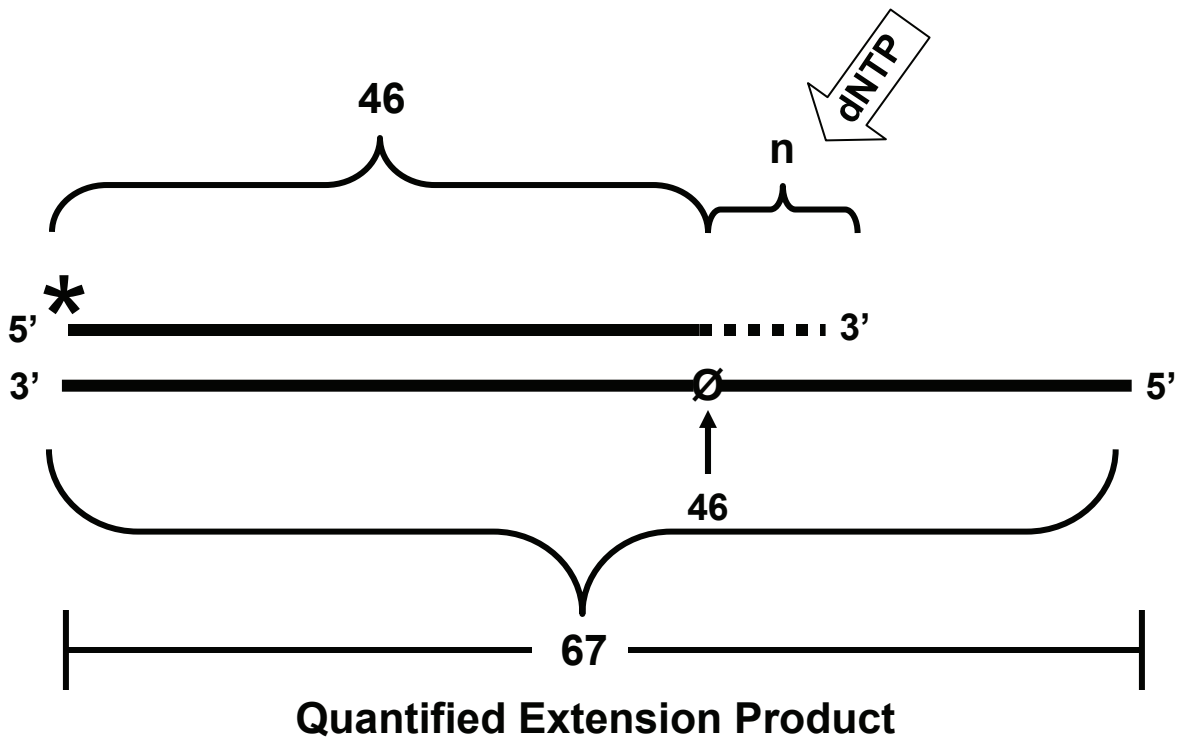
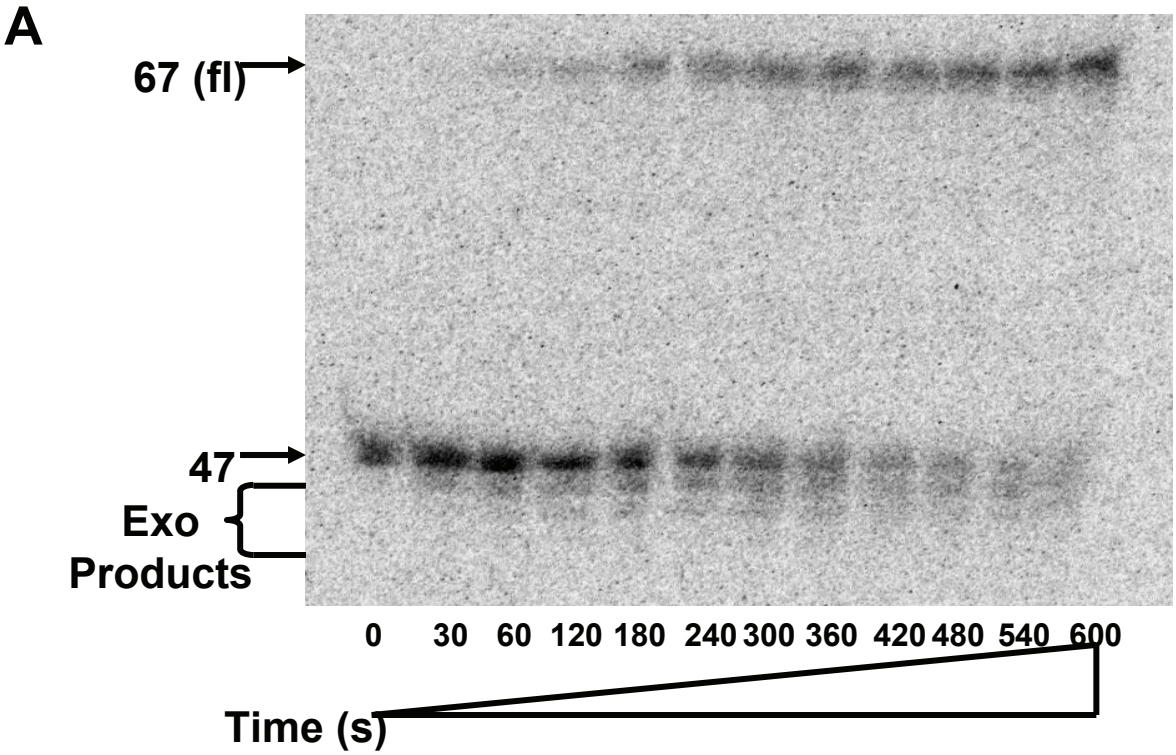



**Fig. 2**

**Figure 2:** Schematic representation of experimental setup. Primers 47, 48, or 49 nucleotides long (n=1,2,and 3 nucleotides downstream of AP site) were 5' end-labeled with [γ-32P-ATP] and annealed to 67 mer templates. Templates contained either a T residue or an AP site analogue (Ø) at the 46$^{th}$ position from the 3' end. All primers contained an A residue at the 46$^{th}$ position. Full length extension products (67 nt) were quantified in each experiment.


A
67 (fl)
47
Exo
Products
0 30 60 120 180 240 300 360 420 480 540 600
Time (s)

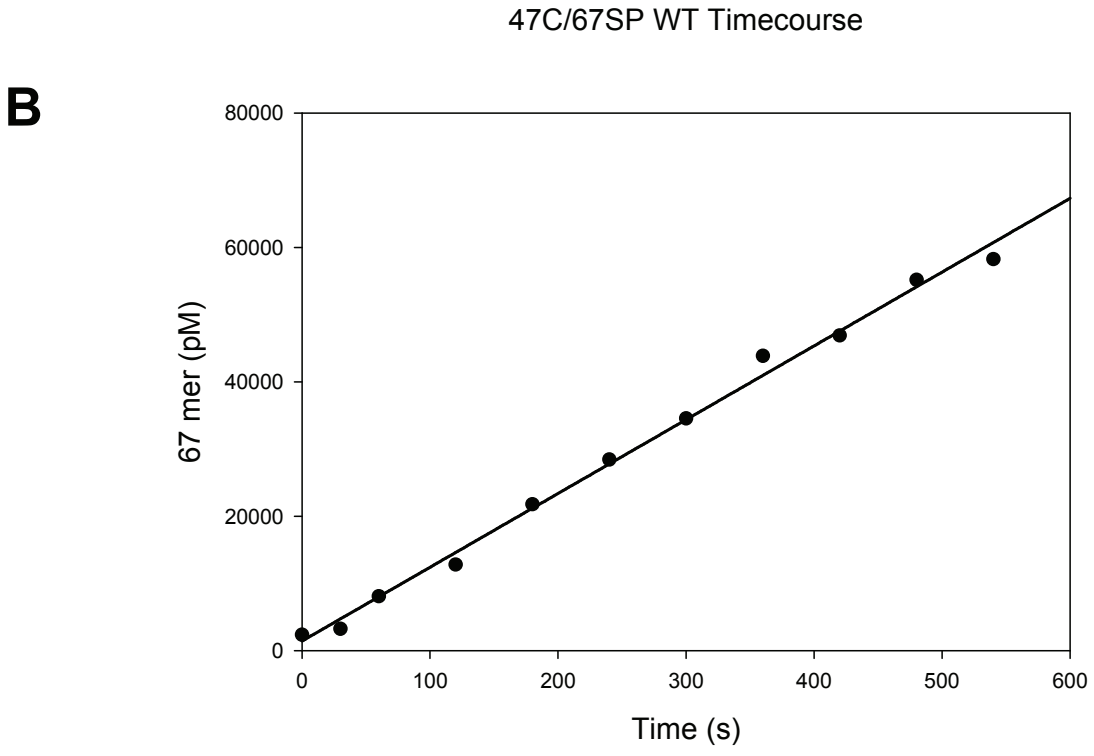
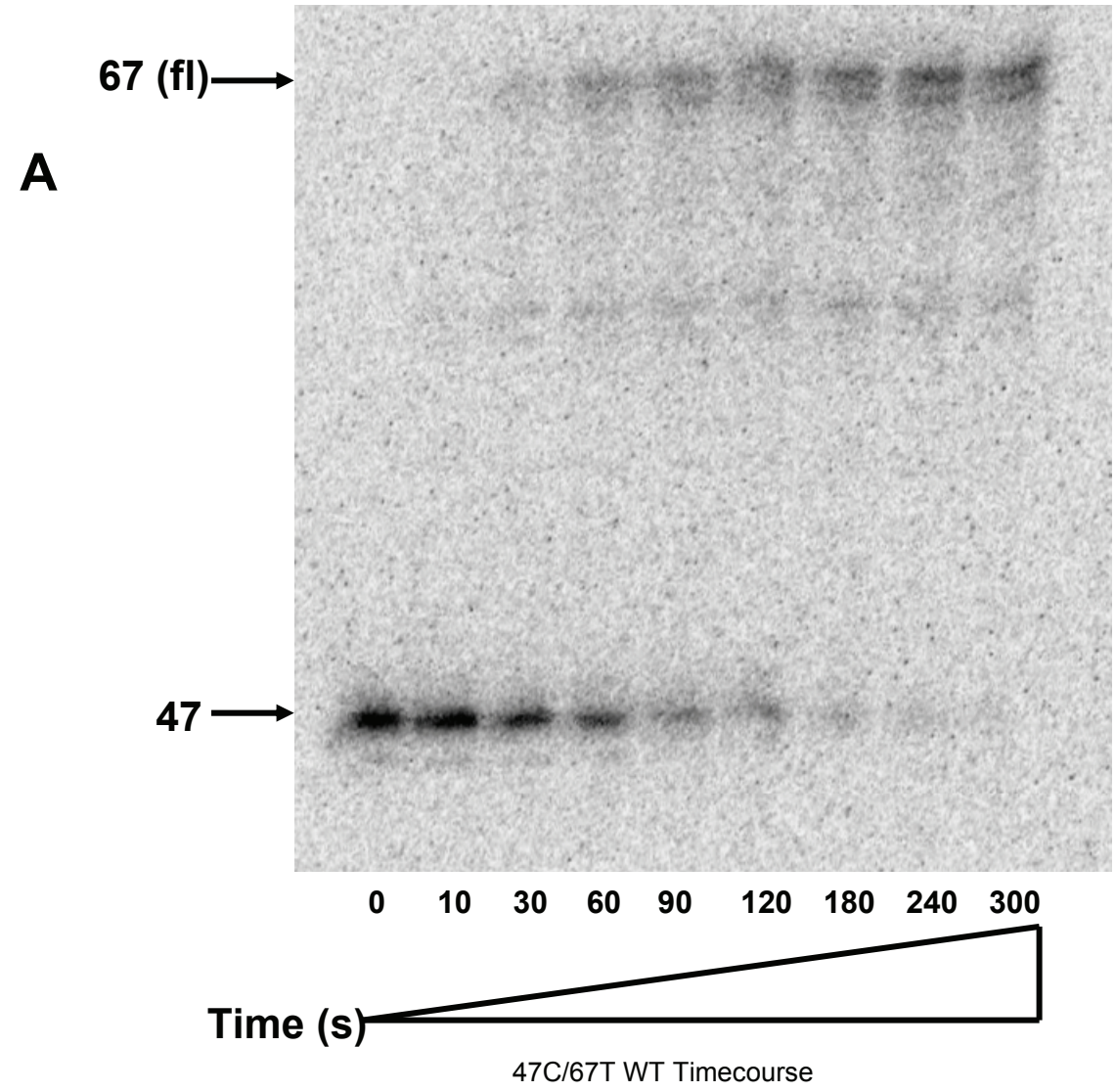

47C/67SP WT Timecourse
B
80000
60000
40000
20000
0
67 mer (pM)
0
100
200
300
400
500
600
Time (s)

**Fig. 3**

**Figure 3:** Determination of kinetics of extension of (+1) primer by WT pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics throughout the time of the experiment.


A
67 (fl)
47
0 10 30 60 90 120 180 240 300
Time (s)
47C/67T WT Timecourse

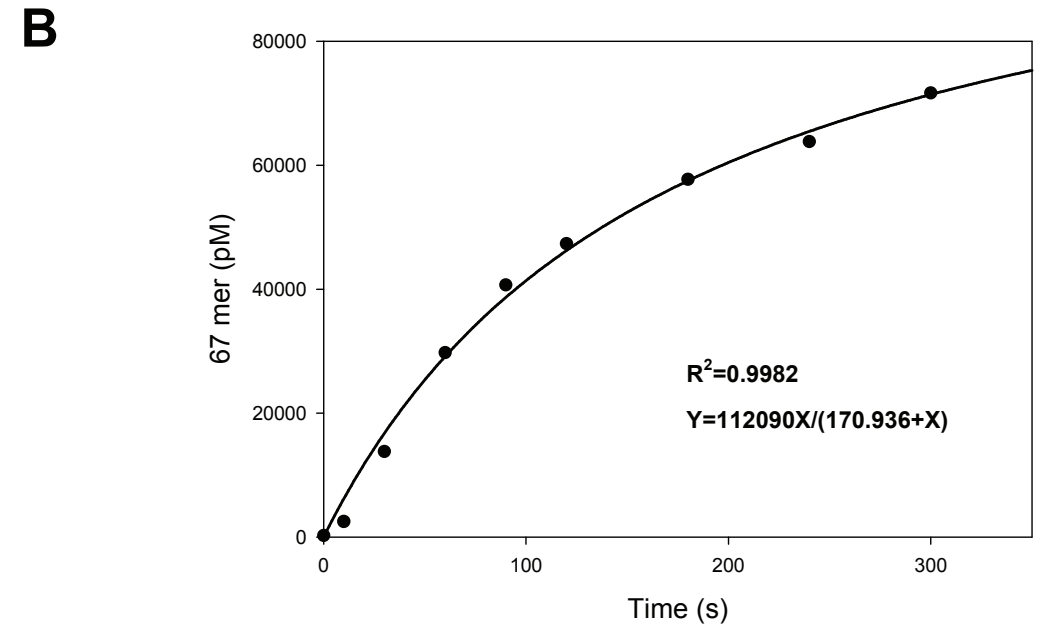
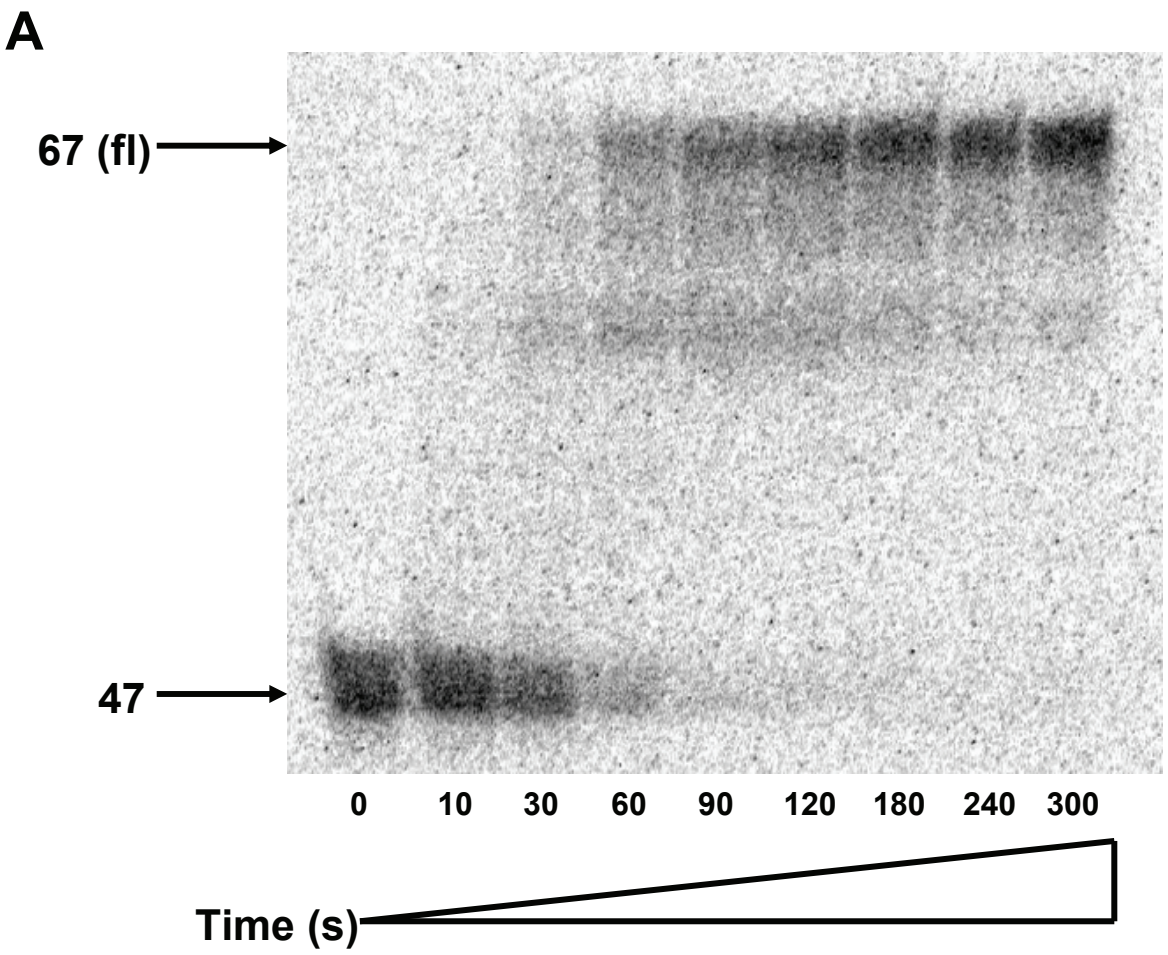

B
80000
60000
40000
20000
0
67 mer (pM)
0
100
200
300
Time (s)
R2=0.9982
Y=112090X/(170.936+X)

**Fig. 4**

**Figure 4:** Determination of kinetics of extension of (+1) primer by WT pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics through 100s.




47C/67T D368A Timecourse

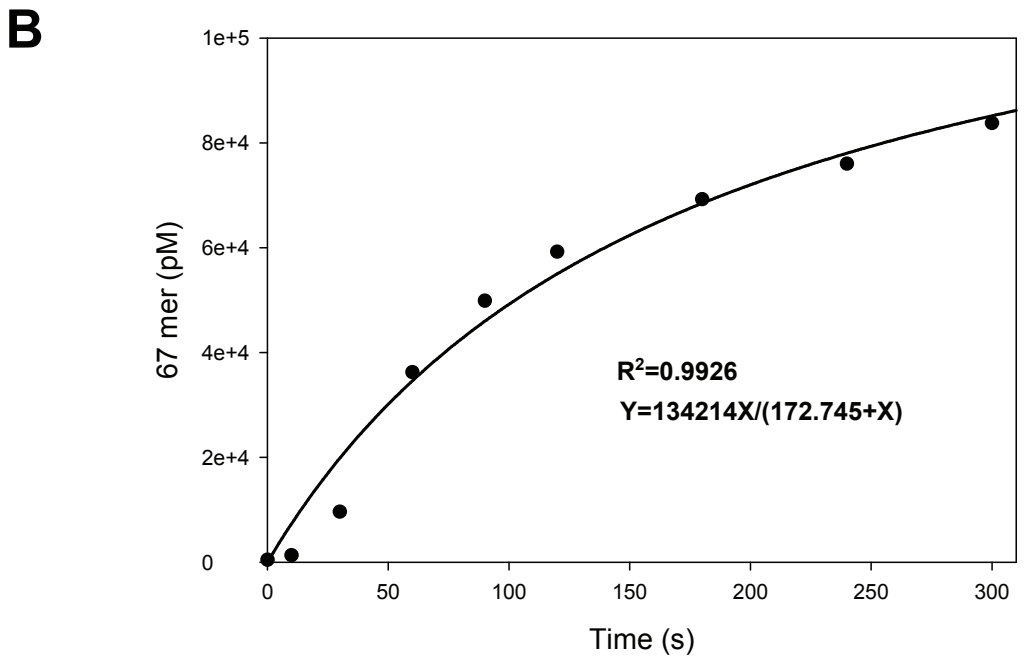
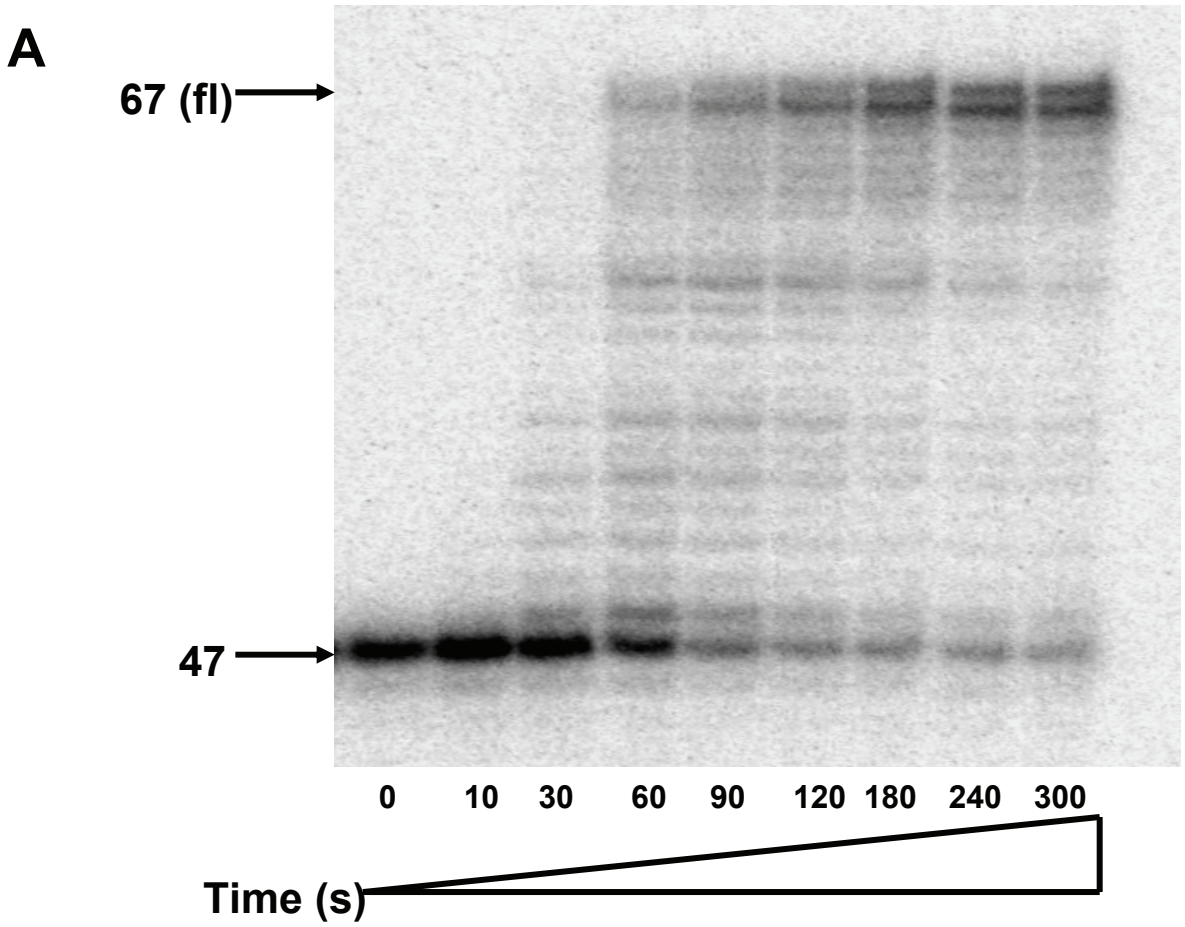



**Fig. 5**

**Figure 5:** Determination of kinetics of extension of (+1) primer by exo$^-$ pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics through 60s.


A
67 (fl)
47
0 10 30 60 90 120 180 240 300
Time (s)

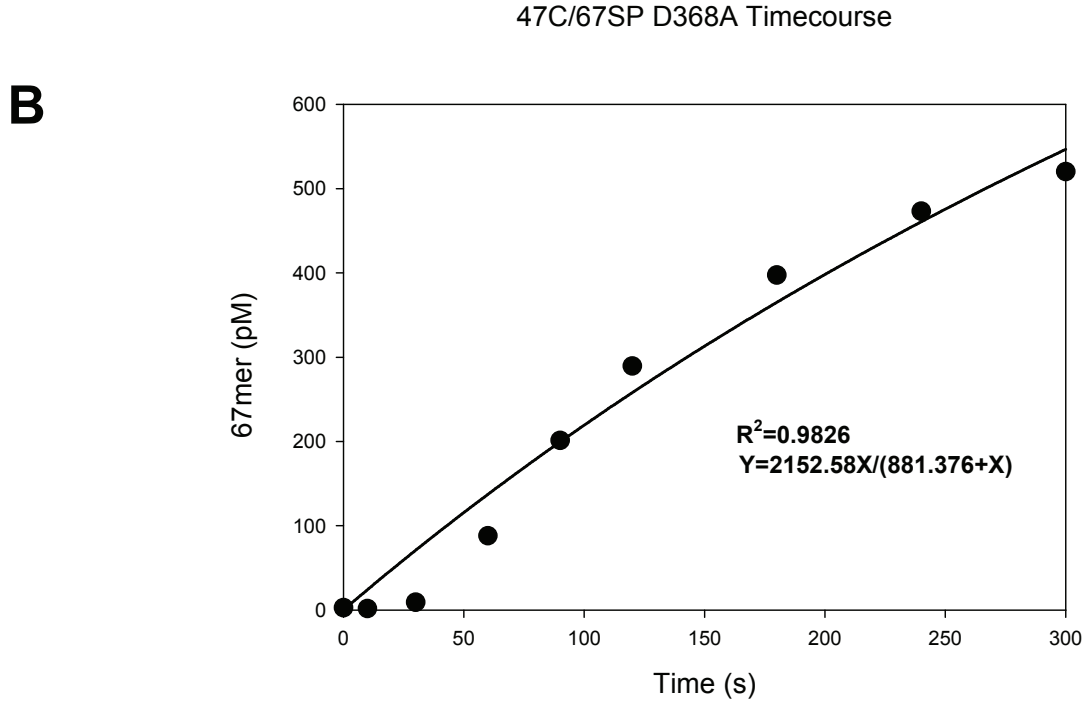

47C/67SP D368A Timecourse
B
67mer (pM)
600
500
400
300
200
100
0
0 50 100 150 200 250 300
Time (s)
R2=0.9826
Y=2152.58X/(881.376+X)

**Fig. 6**

**Figure 6:** Determination of kinetics of extension of (+1) primer by exo$^-$ pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics through 300s.

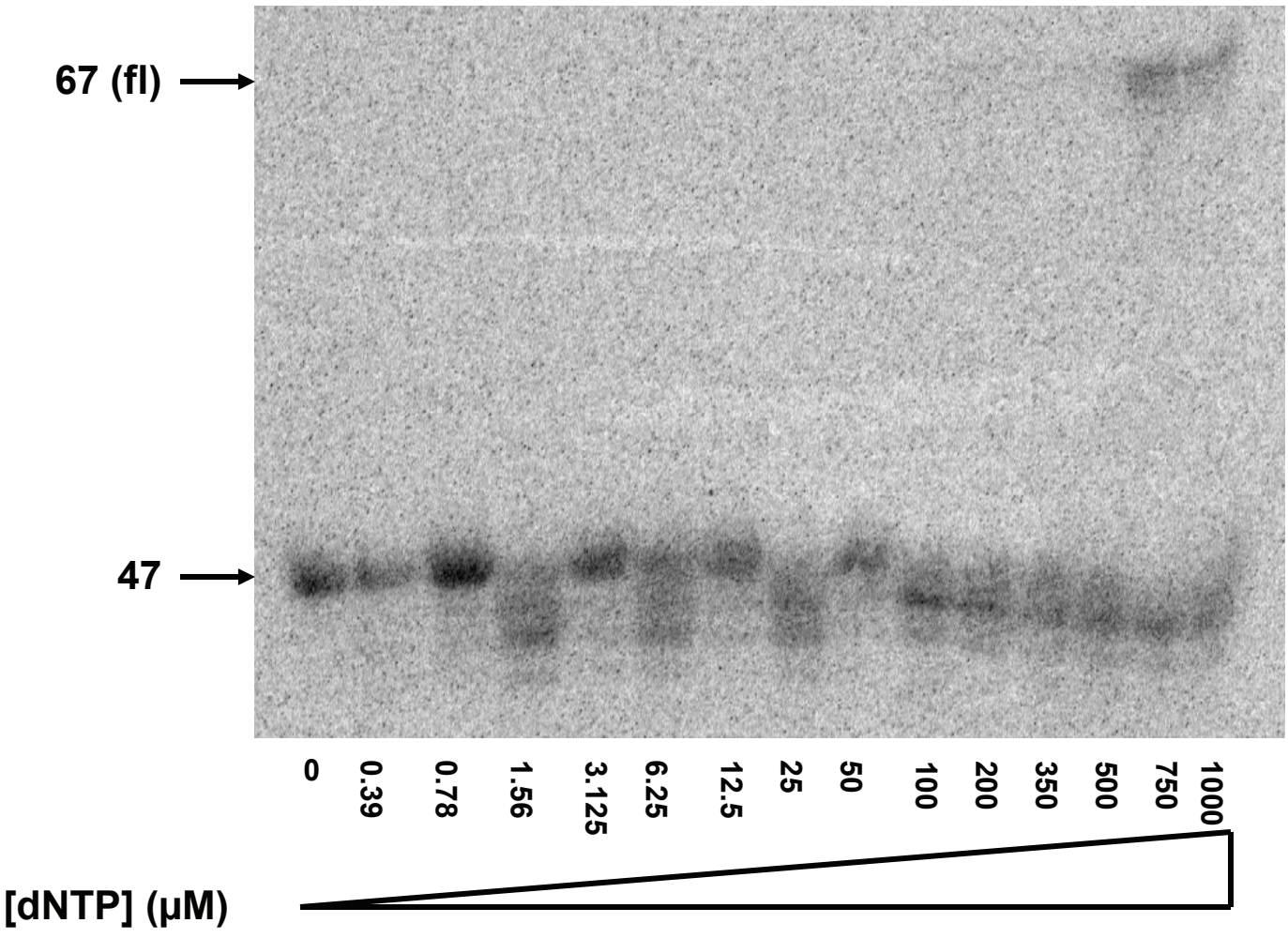
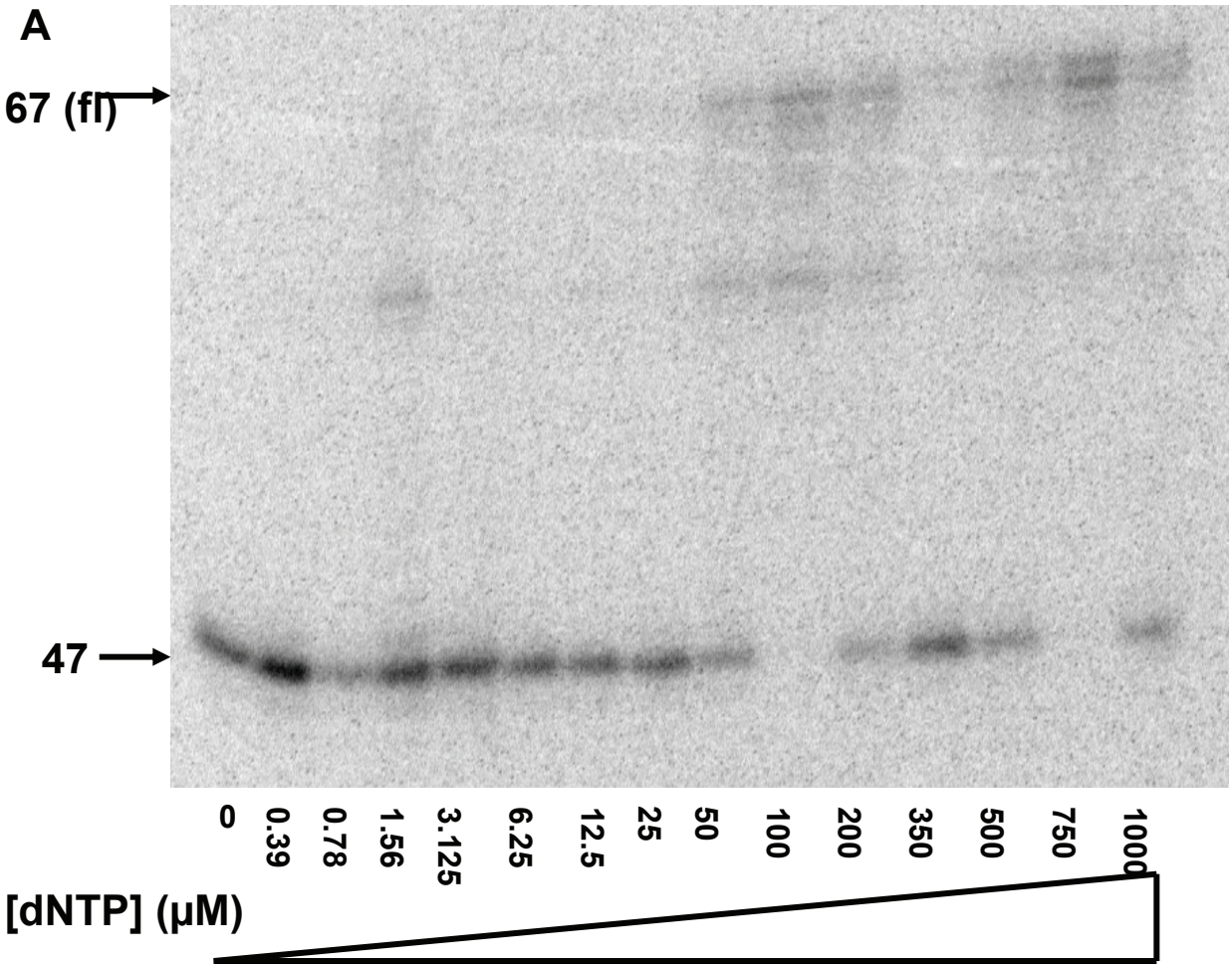

67 (fl)
47
0
0.39
0.78
1.56
3.125
6.25
12.5
25
50
100
200
350
500
750
1000
[dNTP] (μM)

**Fig. 7**

**Figure 7:** Nucleotide concentration dependence assay for WT pol on (+1) damaged template. Reaction was allowed to run for 300s. The amount of extension was unquantifiable.




47C/67T WT Nucleotide Concentration Dependence

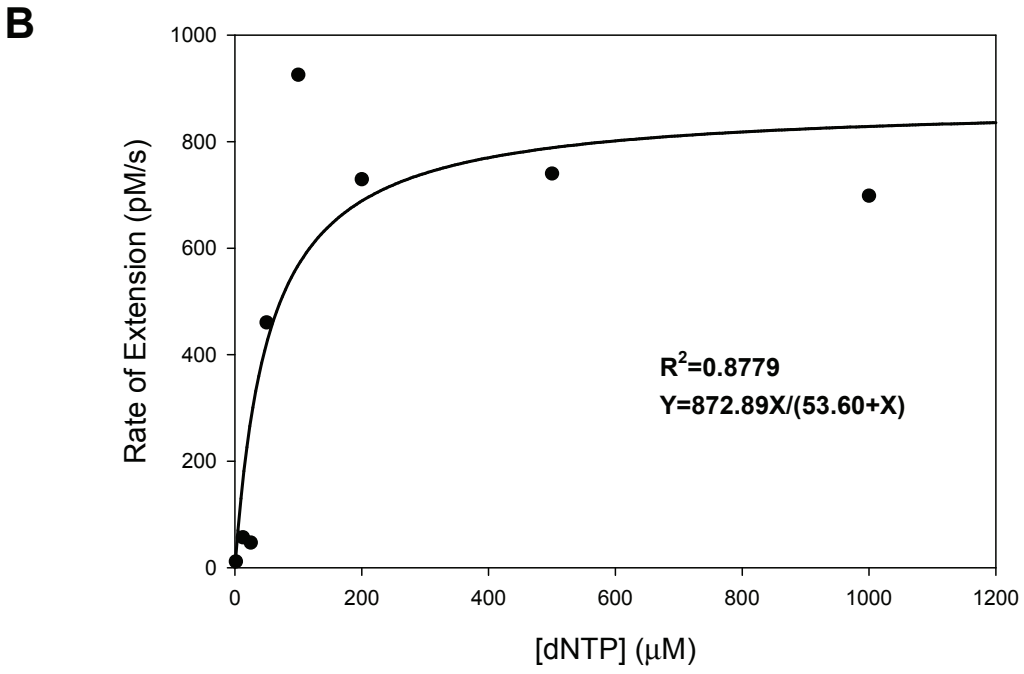
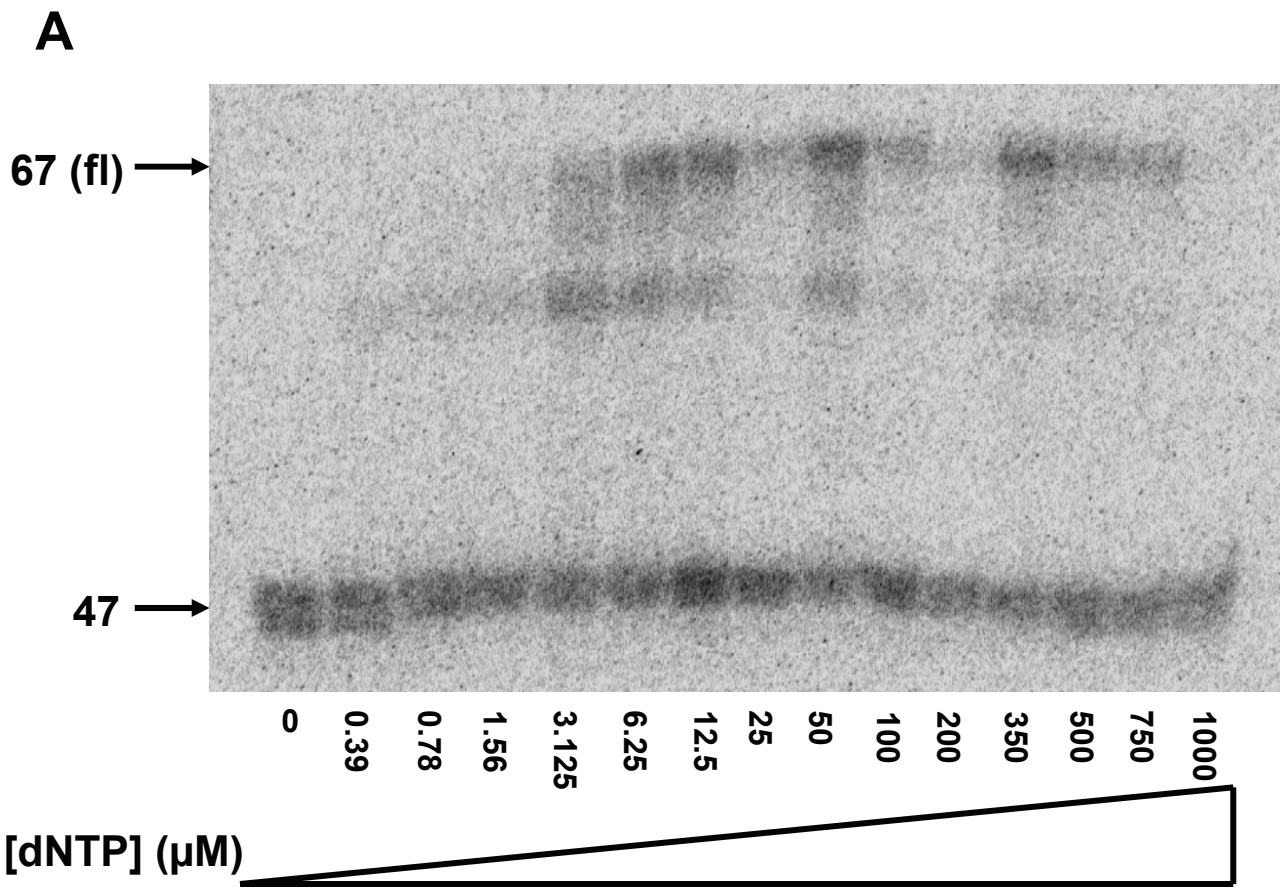



**Fig. 8**

**Figure 8:** Nucleotide concentration dependence assay for WT pol on (+1) undamaged template. Reaction was allowed to run for 60s. Visualization of products (A) and quantification of extension products (B). Data was fit to a hyperbolic curve and efficiency of extension was determined using Equation 1.


A
67 (fl)
47
0
0.39
0.78
1.56
3.125
6.25
12.5
25
50
100
200
350
500
750
1000
[dNTP] (μM)

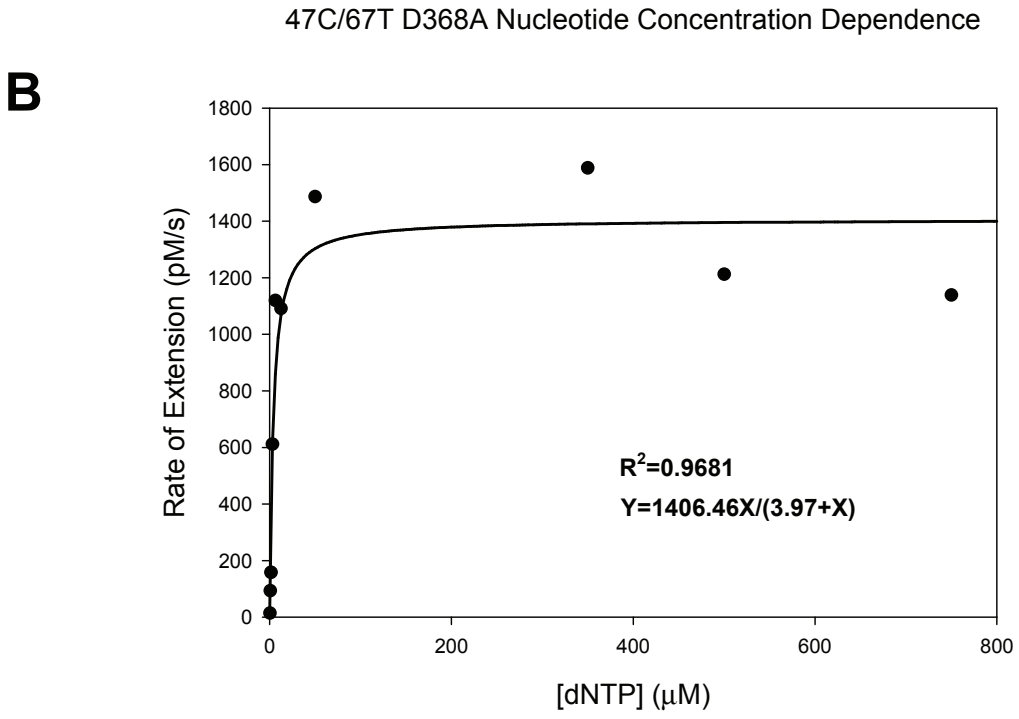
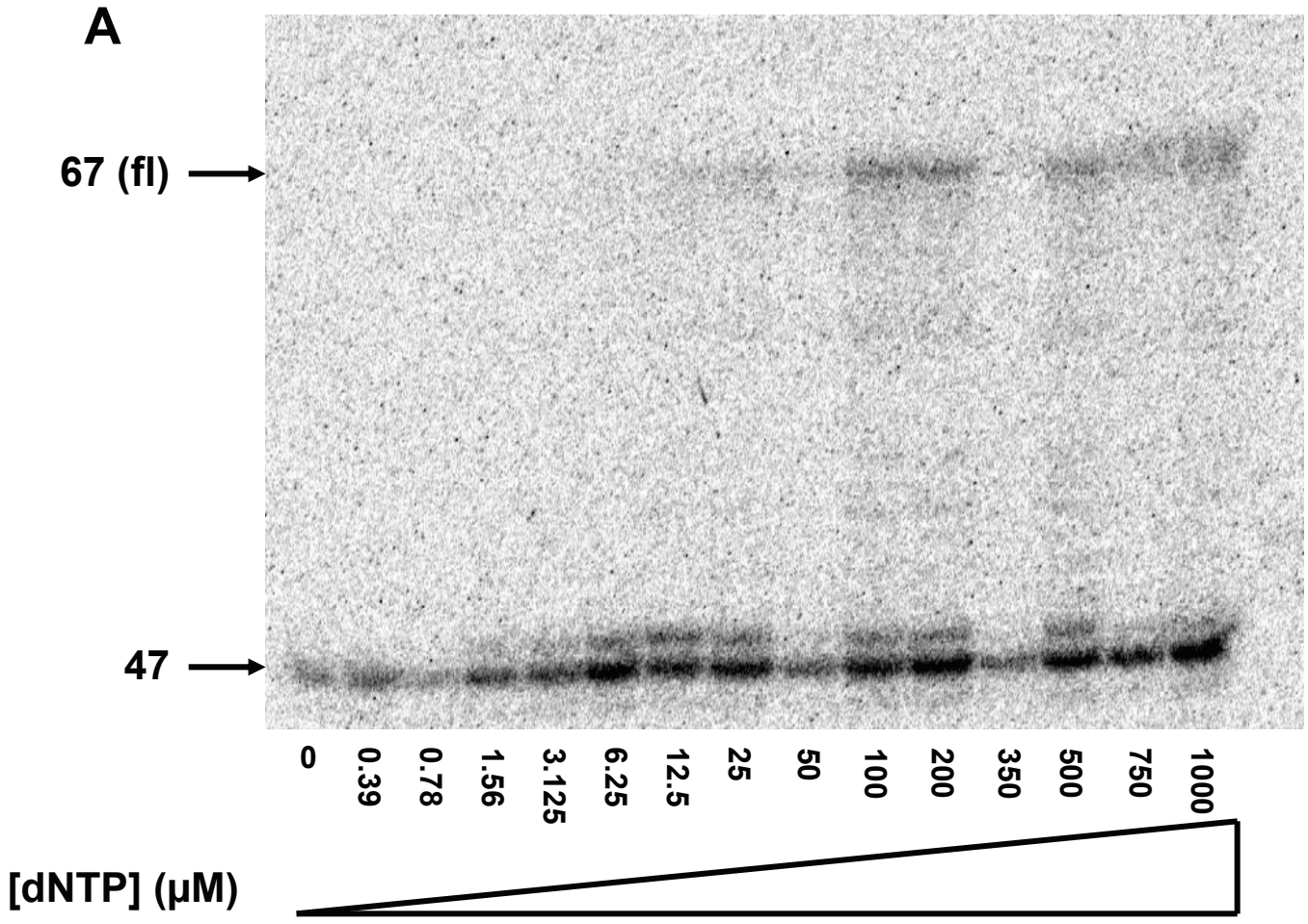

47C/67T D368A Nucleotide Concentration Dependence
B
Rate of Extension (pM/s)
1800
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
[dNTP] (μM)
R2=0.9681
Y=1406.46X/(3.97+X)

**Fig. 9**

**Figure 9:** Nucleotide concentration dependence assay for exo- pol on (+1) undamaged template. Reaction was allowed to run for 60s. Visualization of products (A) and quantification of extension products (B). Data was fit to a hyperbolic curve and efficiency of extension was determined using Equation 1.




47C/67SP D368A Nucleotide Concentration Dependence

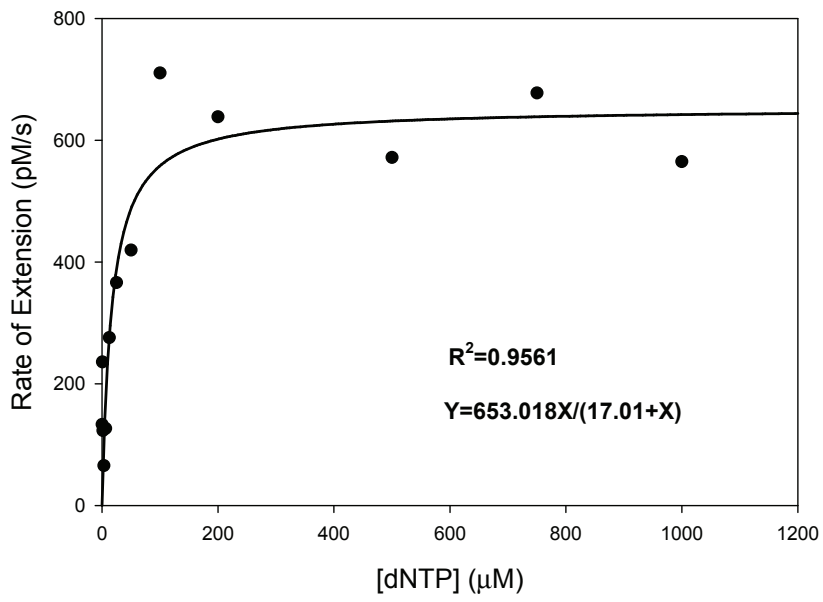
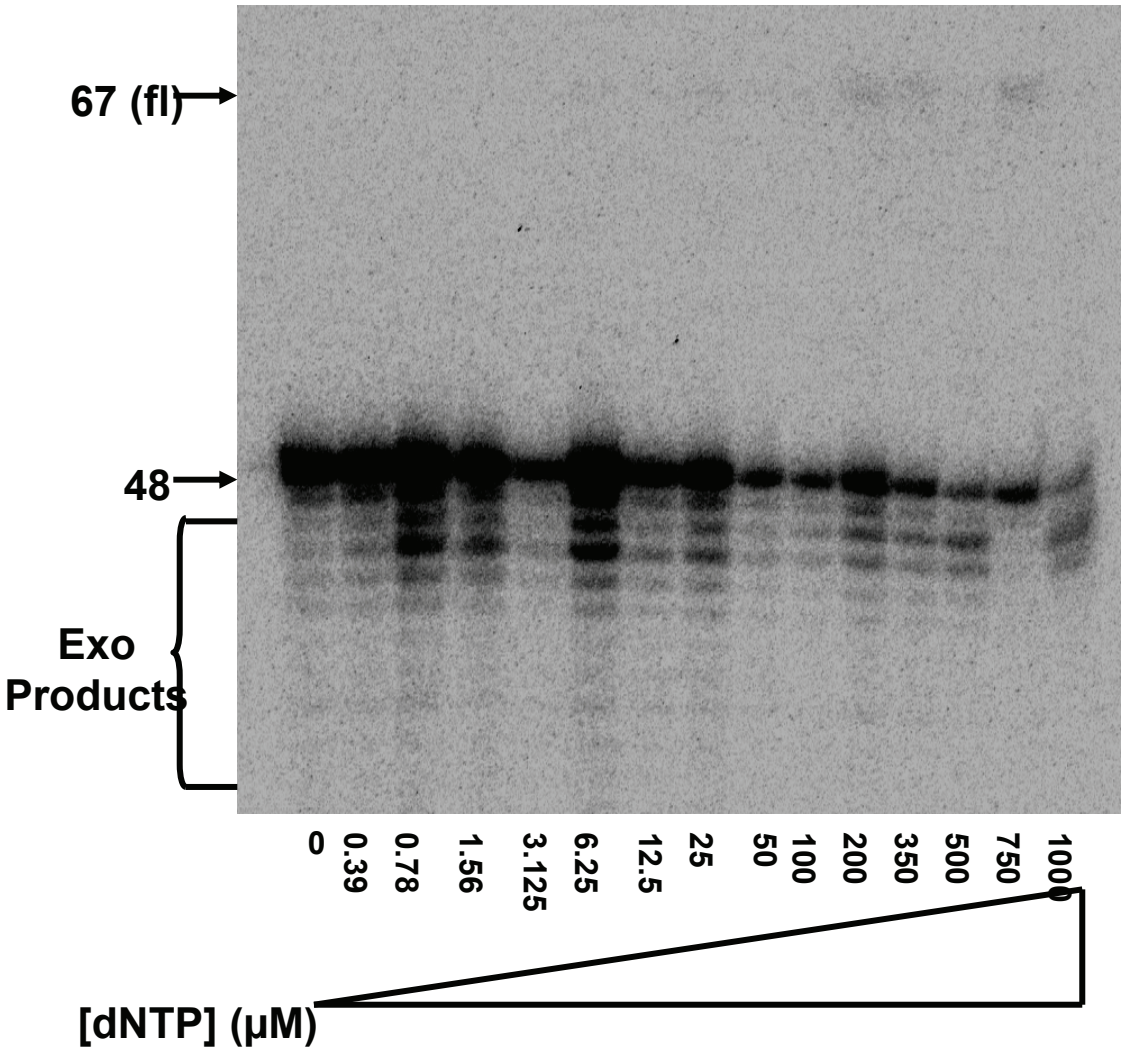



**Fig. 10**

**Figure 10:** Nucleotide concentration dependence assay for exo- pol on (+1) damaged template. Reaction was allowed to run for 300s. Visualization of products (A) and quantification of extension products (B). Data was fit to a hyperbolic curve and efficiency of extension was determined using Equation 1.




48C/67SP WT Nucleotide Concentration Dependence

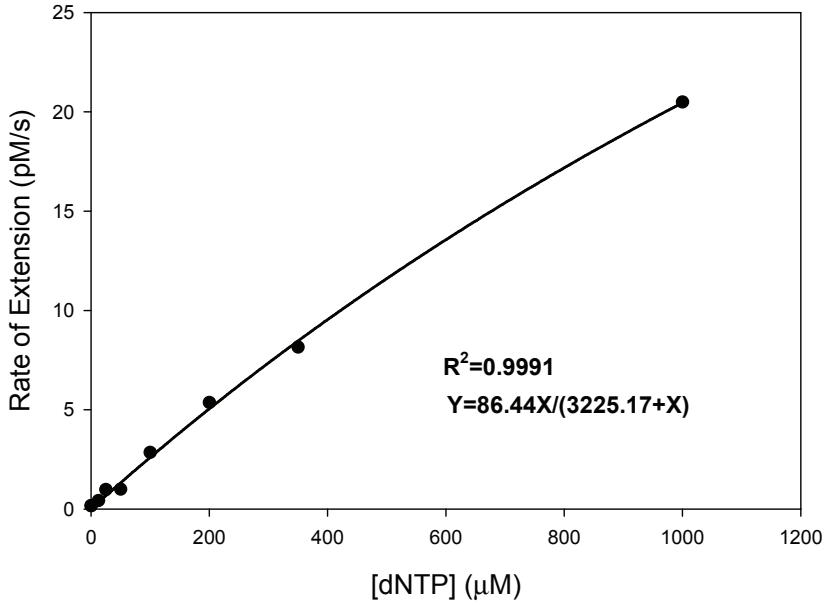
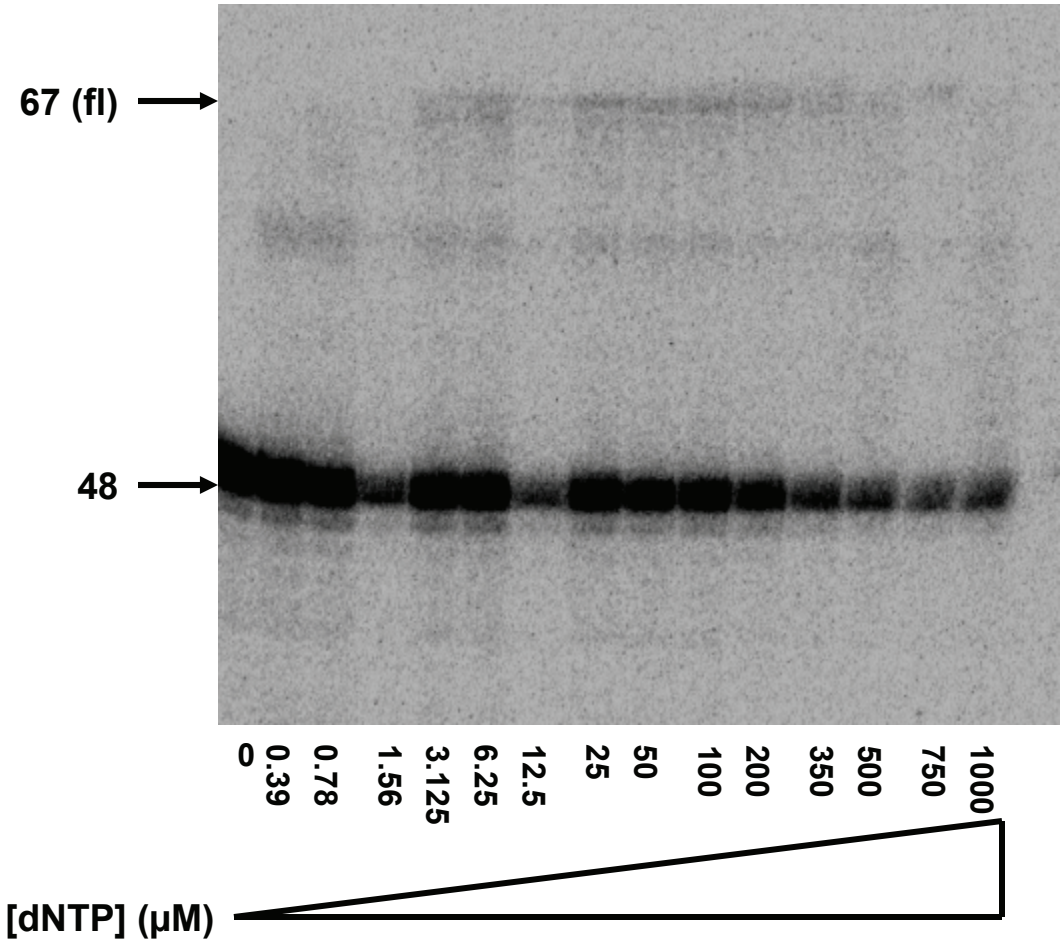



**Fig. 11**

**Figure 11:** Nucleotide concentration dependence assay for WT pol on (+2) damaged template. Reaction was allowed to run for 300s. Visualization of products (A) and quantification of extension products (B). Data was fit to a hyperbolic curve and efficiency of extension was determined using Equation 1.

**A**


67 (fl)
48
0
0.39
0.78
1.56
3.125
6.25
12.5
25
50
100
200
350
500
750
1000
[dNTP] (μM)

**B**

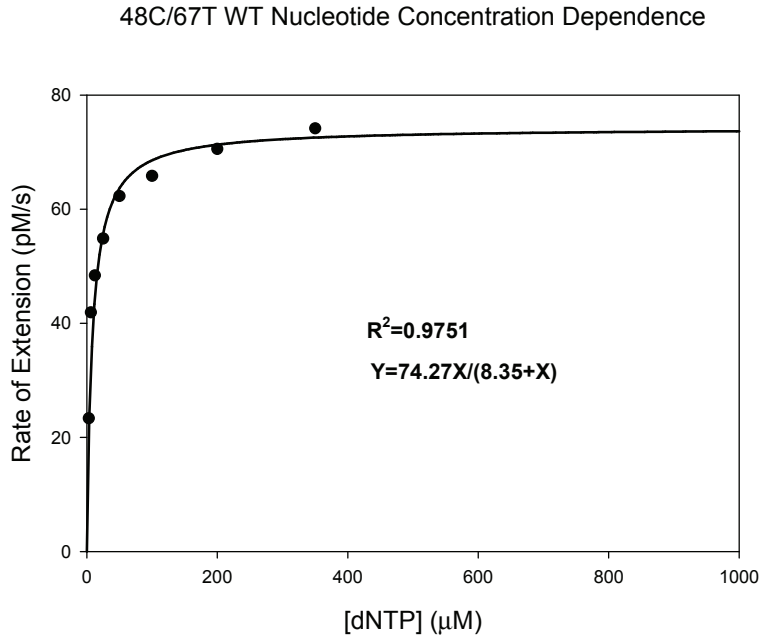
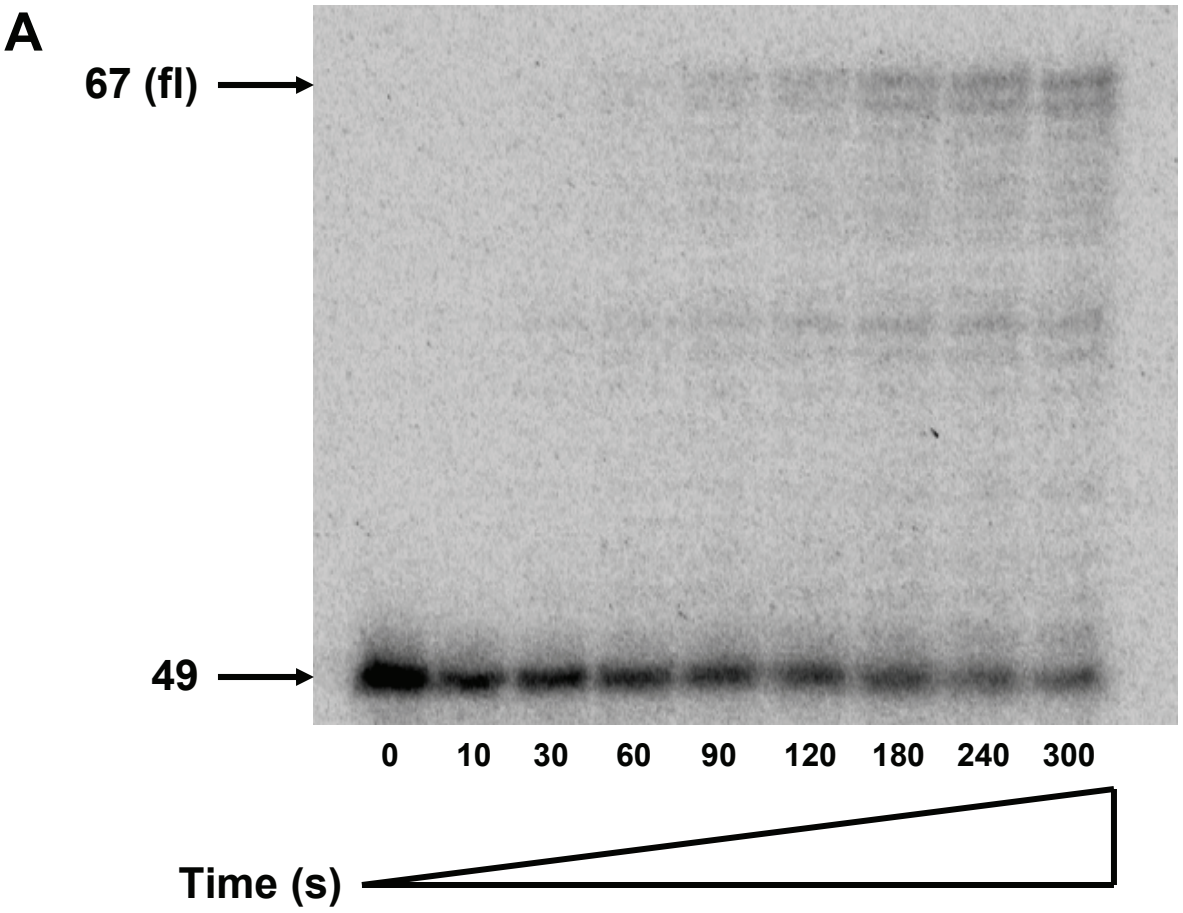

48C/67T WT Nucleotide Concentration Dependence
Rate of Extension (pM/s)
[dNTP] (μM)
R2=0.9751
Y=74.27X/(8.35+X)
0
20
40
60
80
0
200
400
600
800
1000

**Fig. 12**

**Figure 12:** Nucleotide concentration dependence assay for WT pol on (+2) undamaged template. Reaction was allowed to run for 60s. Visualization of products (A) and quantification of extension products (B). Data was fit to a hyperbolic curve and efficiency of extension was determined using Equation 1.


A
67 (fl)
49
0 10 30 60 90 120 180 240 300
Time (s)

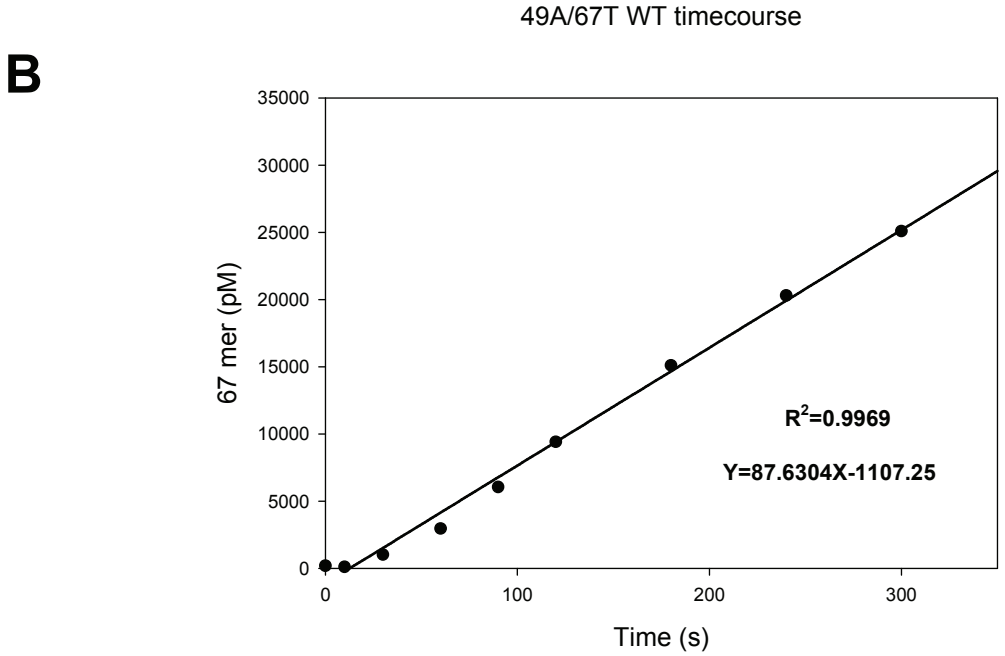
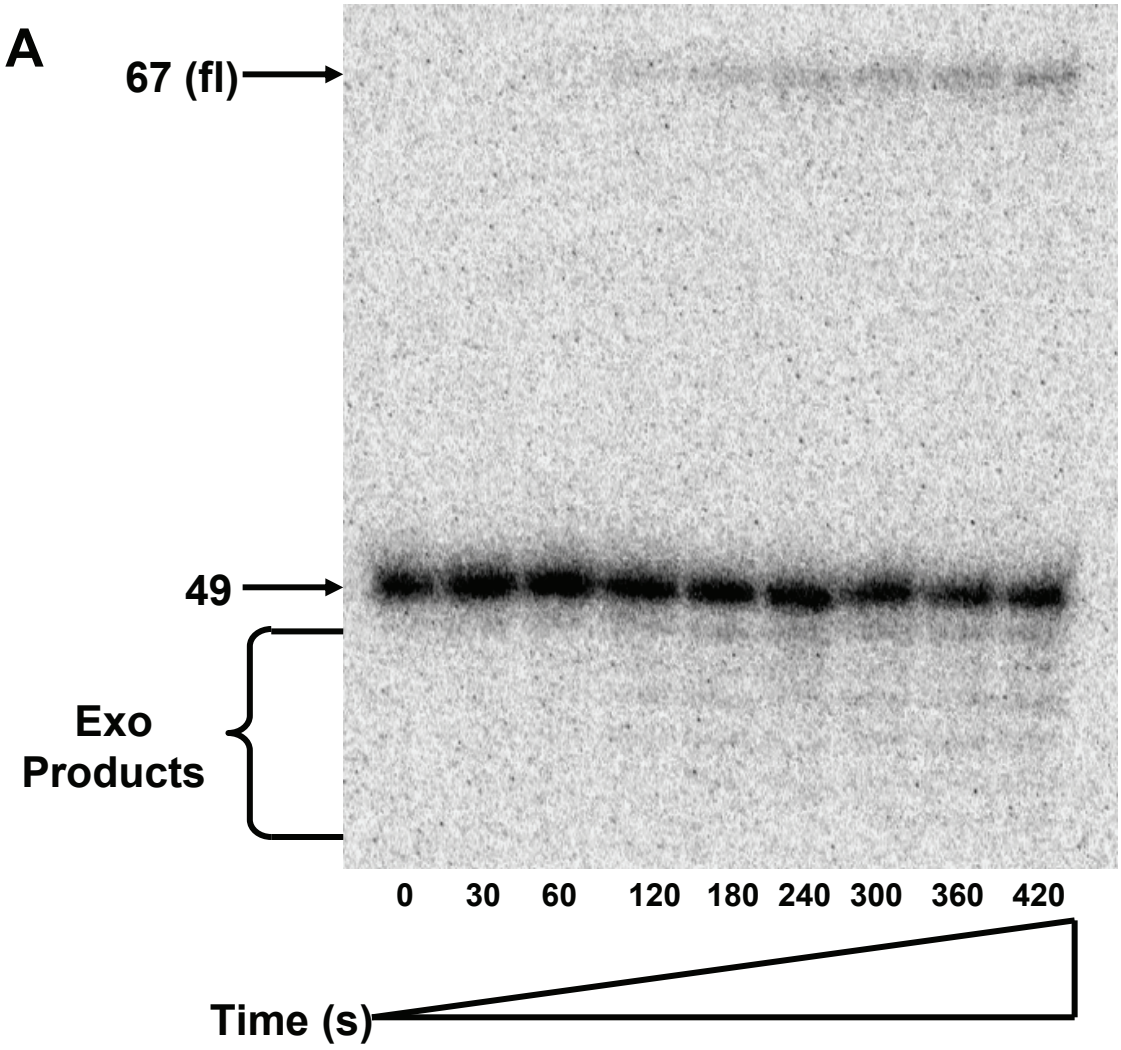

B
49A/67T WT timecourse
67 mer (pM)
35000
30000
25000
20000
15000
10000
5000
0
0
100
200
300
Time (s)
R2=0.9969
Y=87.6304X-1107.25

**Fig. 13**

**Figure 13:** Determination of kinetics of extension of (+3) primer by WT pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics throughout the time of the experiment.


A
67 (fl)
49
Exo
Products
0 30 60 120 180 240 300 360 420
Time (s)
49A/67SP WT timecourse

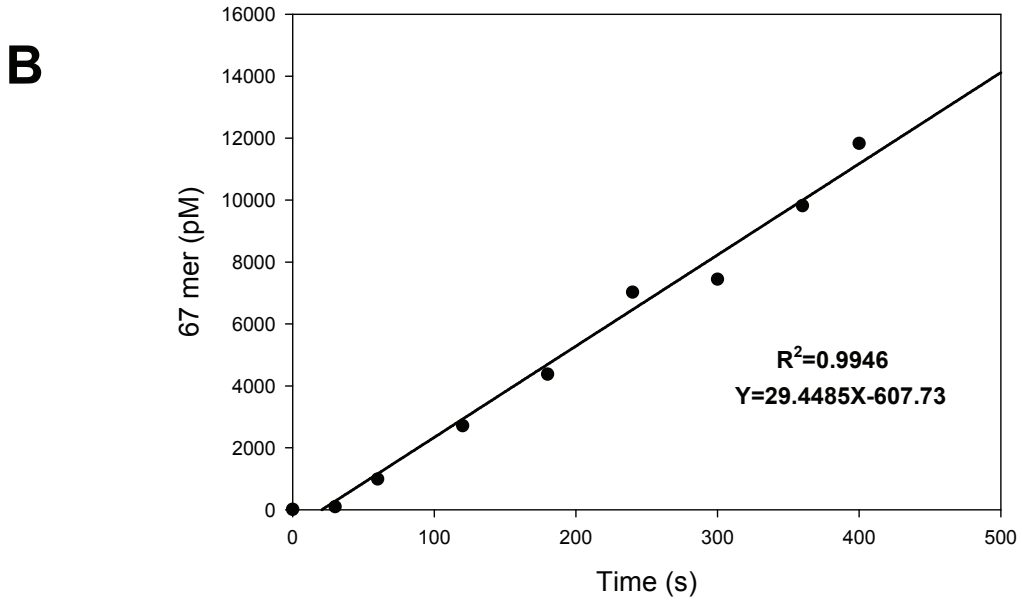
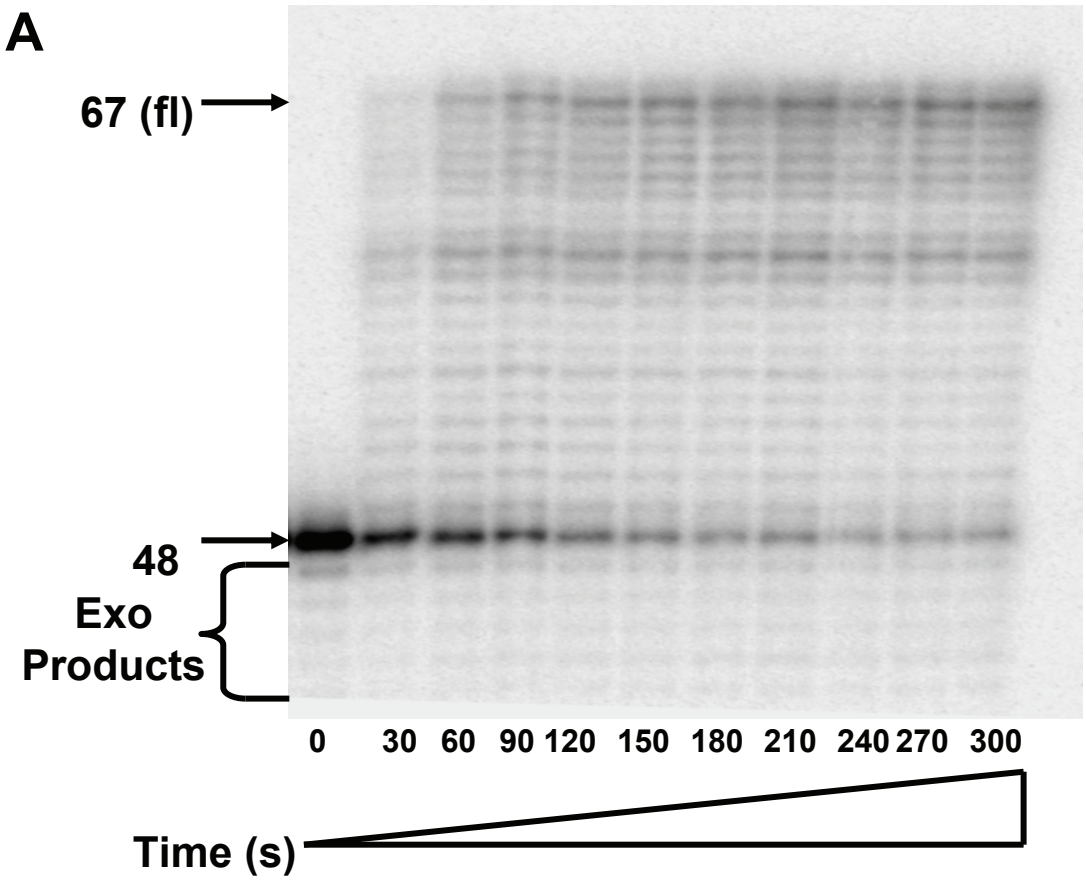

B
16000
14000
12000
10000
8000
6000
4000
2000
0
67 mer (pM)
0
100
200
300
400
500
Time (s)
R2=0.9946
Y=29.4485X-607.73

**Fig. 14**

**Figure 14:** Determination of kinetics of extension of (+3) primer by WT pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics throughout the time of the experiment.




48C/67T WT Extension

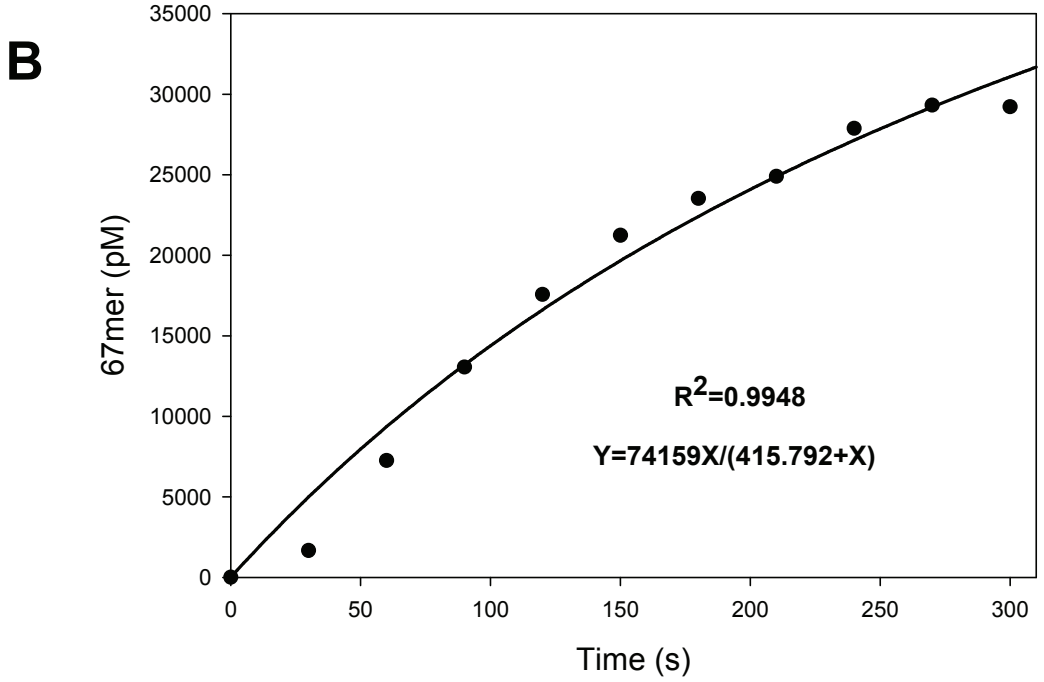
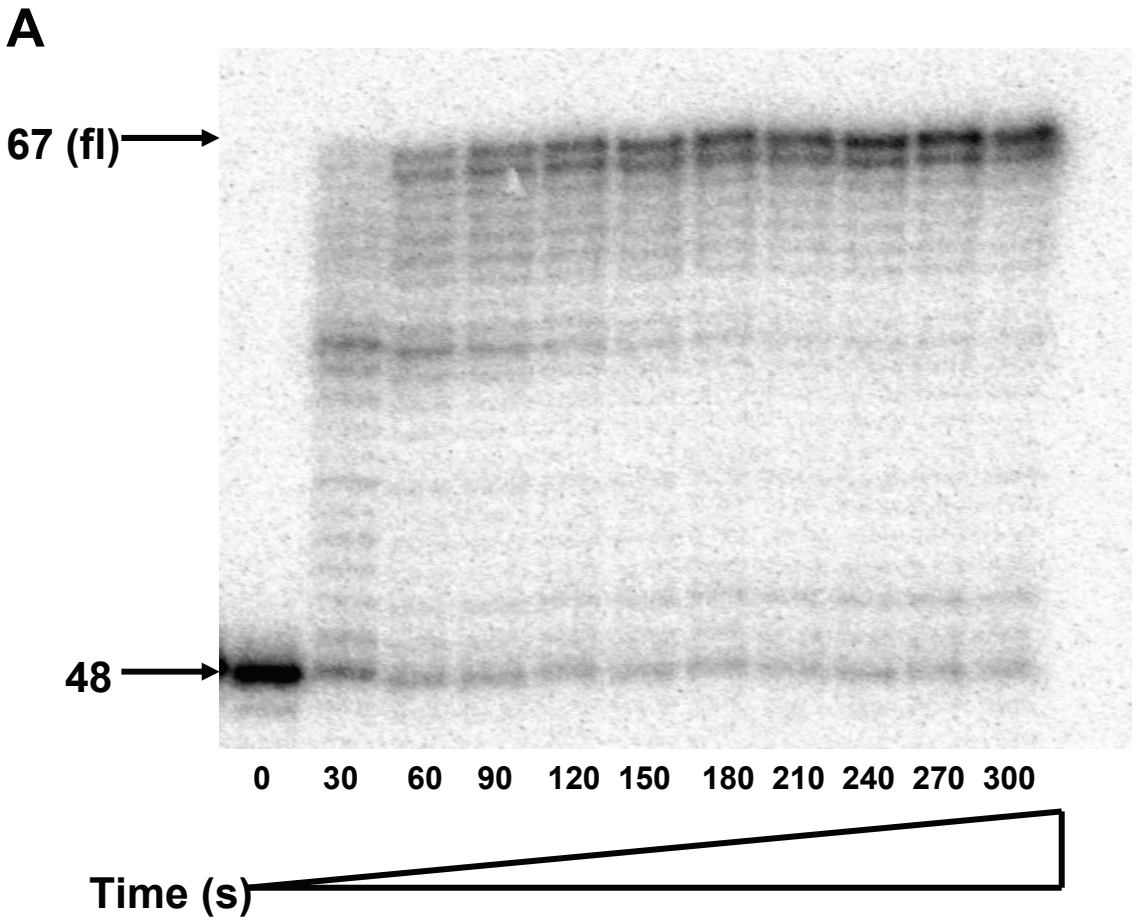



**Fig. 15**

**Figure 15:** Determination of kinetics of extension of (+2) primer by WT pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics 120s


A
67 (fl)
48
0 30 60 90 120 150 180 210 240 270 300
Time (s)
48C/67T D368A Extension

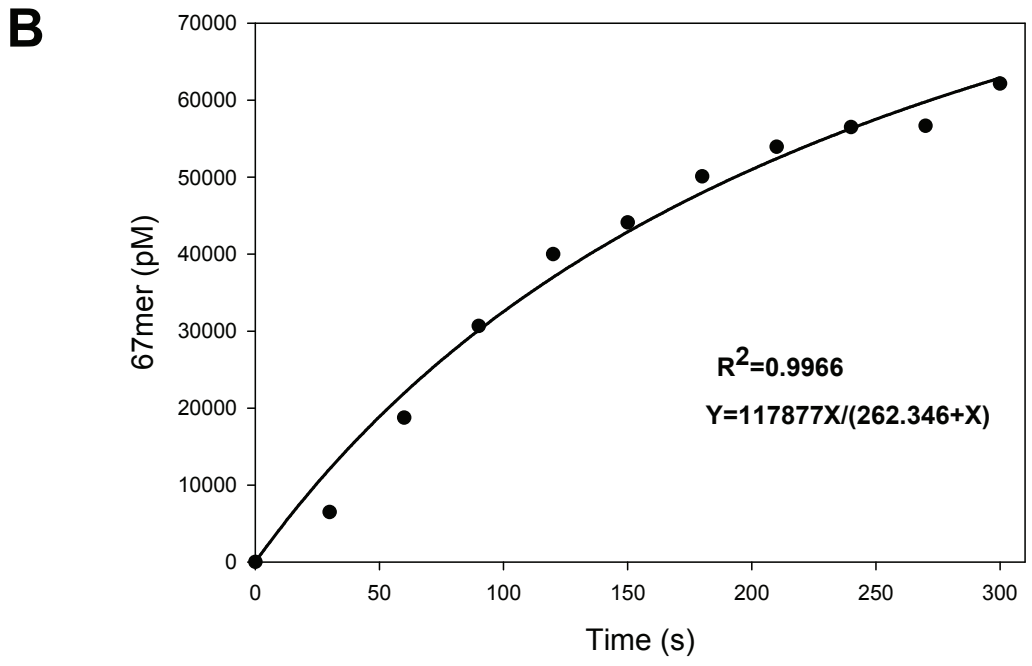
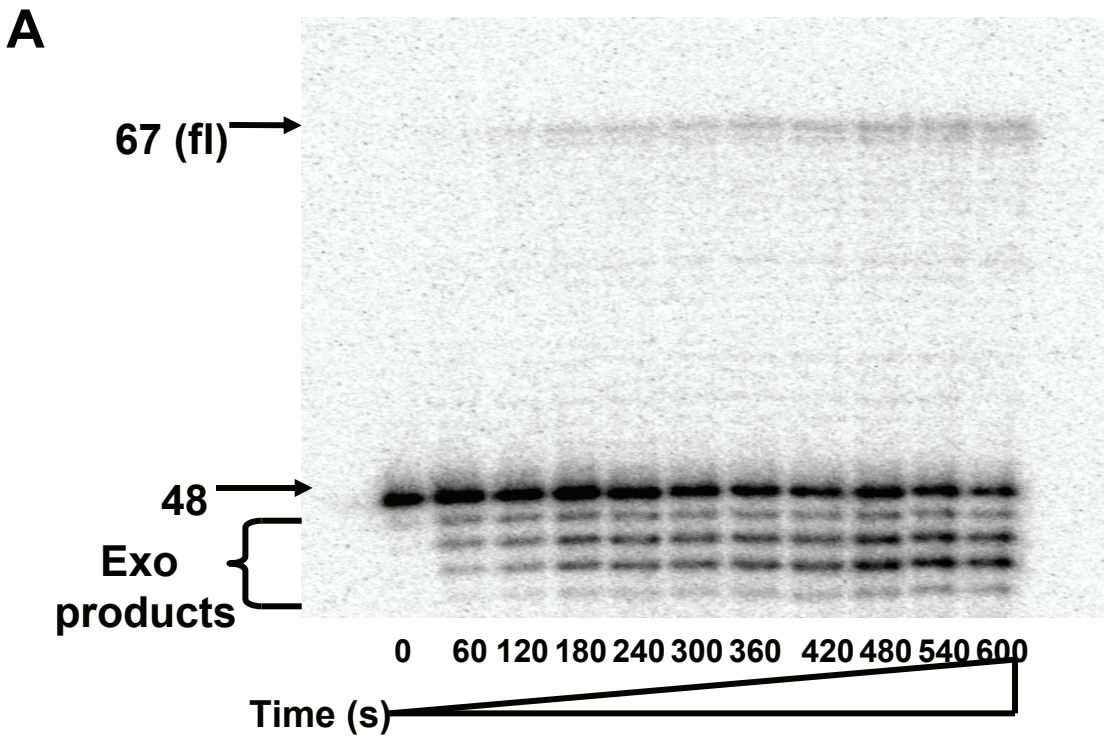

B
70000
60000
50000
40000
30000
20000
10000
0
67mer (pM)
0 50 100 150 200 250 300
Time (s)
R2=0.9966
Y=117877X/(262.346+X)

**Fig. 16**

**Figure 16:** Determination of kinetics of extension of (+2) primer by exo$^-$ pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics 120s


A
67 (fl)
48
Exo
products
0 60 120 180 240 300 360 420 480 540 600
Time (s)

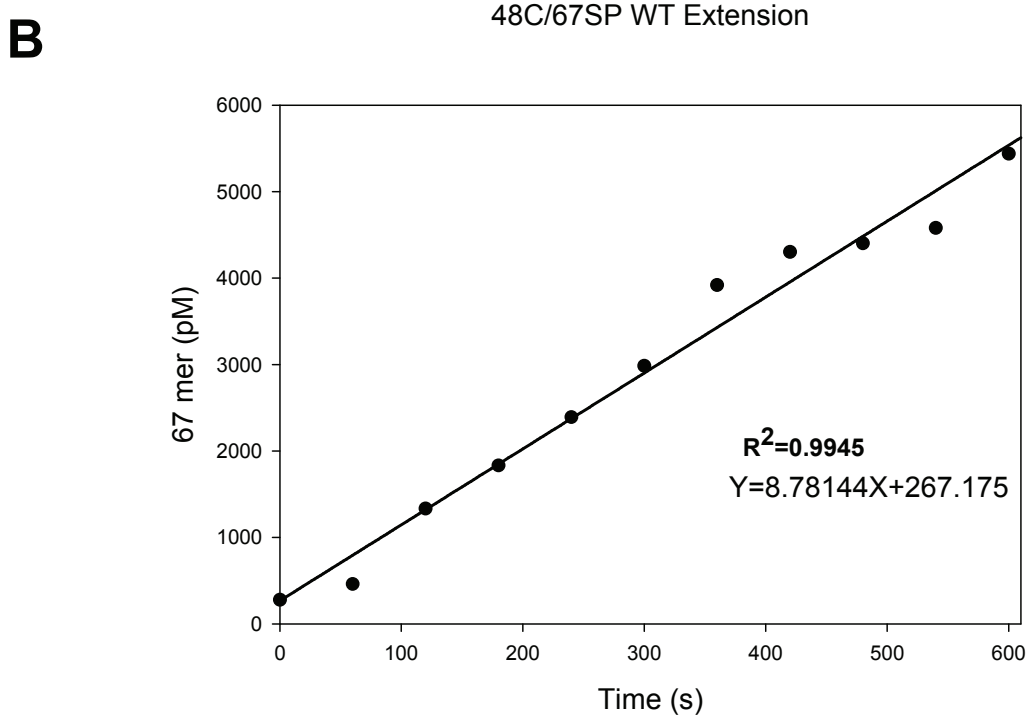
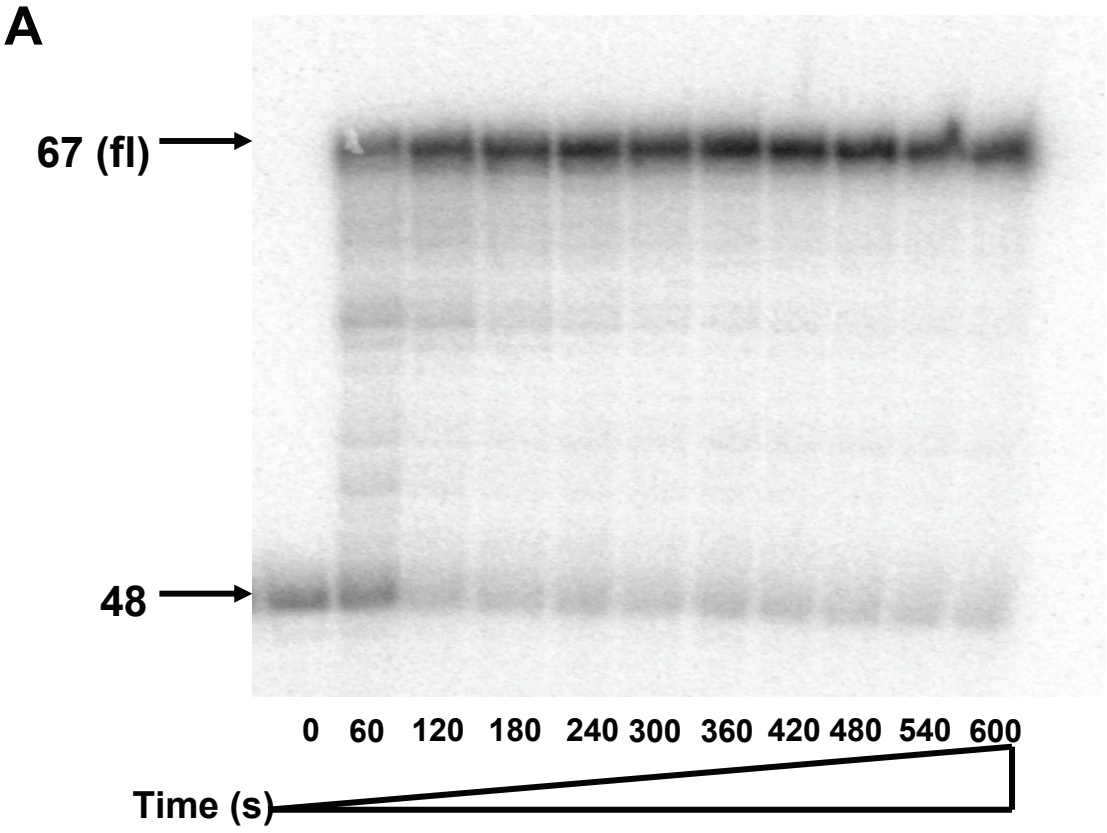

B
48C/67SP WT Extension
67 mer (pM)
Time (s)
R2=0.9945
Y=8.78144X+267.175
0
1000
2000
3000
4000
5000
6000
0
100
200
300
400
500
600

**Fig. 17**

**Figure 17:** Determination of kinetics of extension of (+2) primer by WT pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics throughout the time of the experiement.


A
67 (fl)
48
0 60 120 180 240 300 360 420 480 540 600
Time (s)

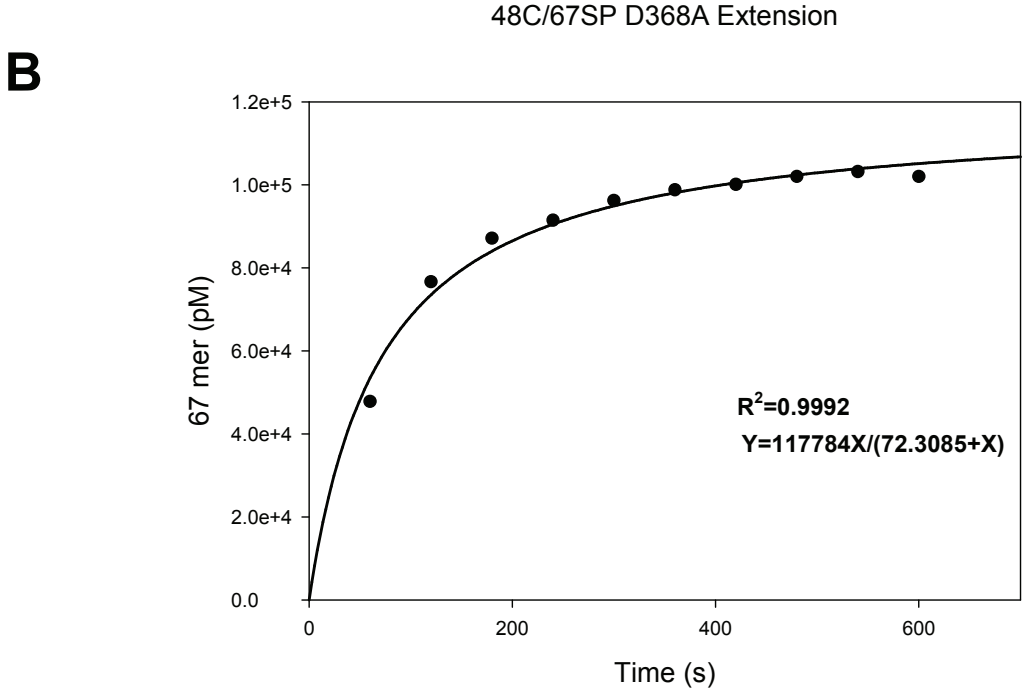
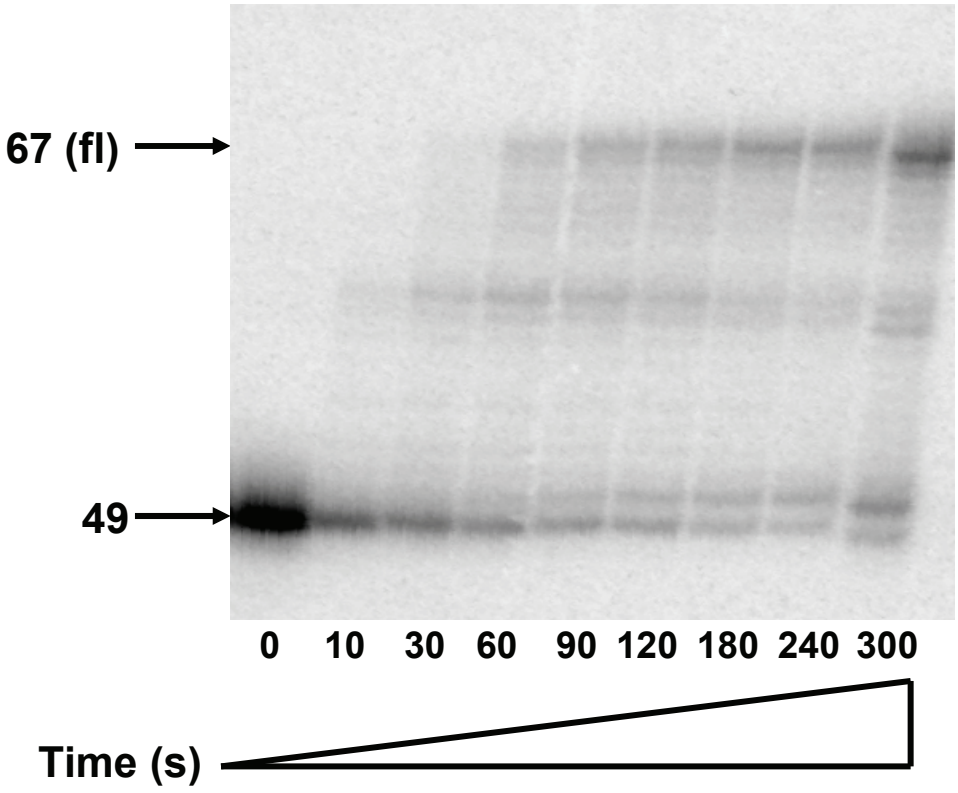

B
48C/67SP D368A Extension
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
67 mer (pM)
0
200
400
600
Time (s)
R2=0.9992
Y=117784X/(72.3085+X)

**Fig. 18**

**Figure 18:** Determination of kinetics of extension of (+2) primer by exo$^-$ pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics through 120s.

**A**


67 (fl)
49
0 10 30 60 90 120 180 240 300
Time (s)

**B**

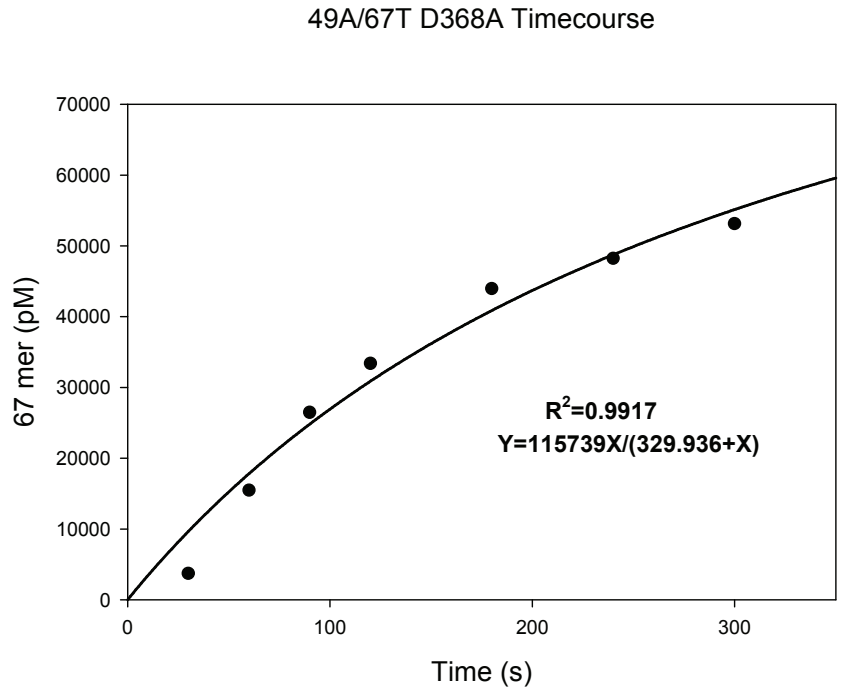
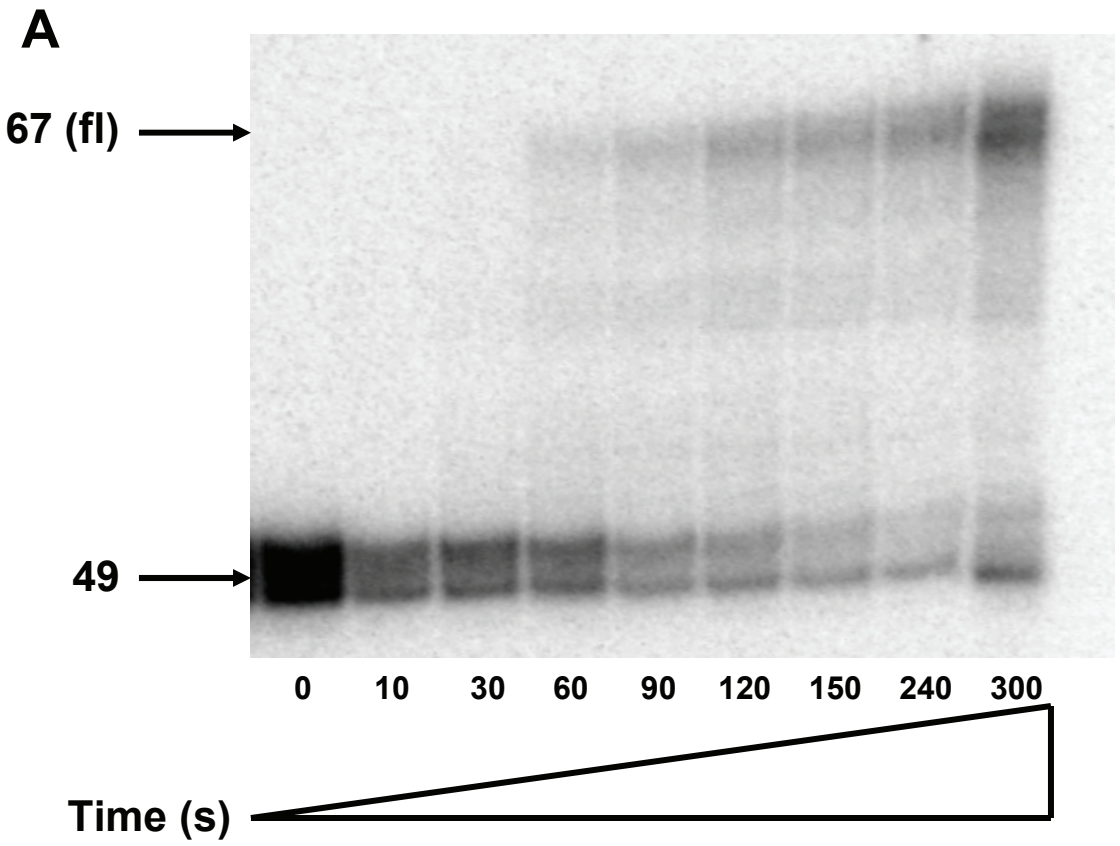

49A/67T D368A Timecourse
70000
60000
50000
40000
30000
20000
10000
0
67 mer (pM)
0
100
200
300
Time (s)
R2=0.9917
Y=115739X/(329.936+X)

**Fig. 19**

**Figure 19:** Determination of kinetics of extension of (+3) primer by exo$^-$ pol on undamaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics through 180s.




49A/67SP D368A Timecourse

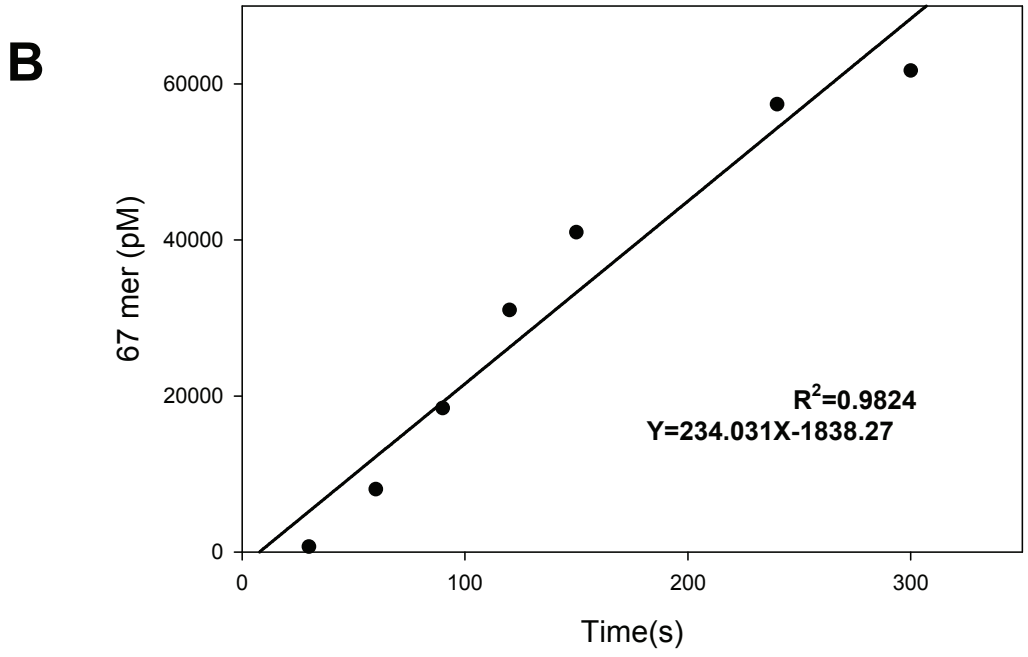
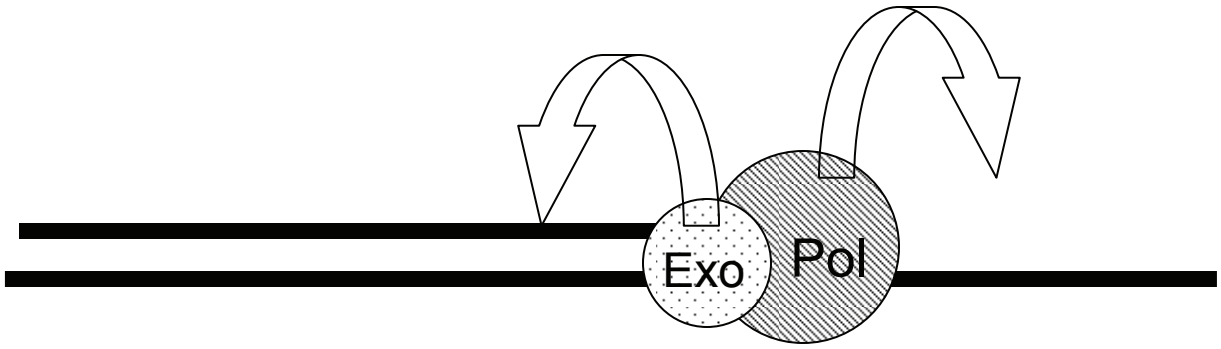
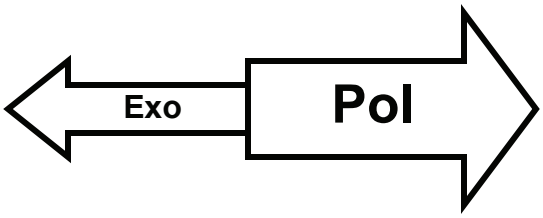



**Fig. 20**

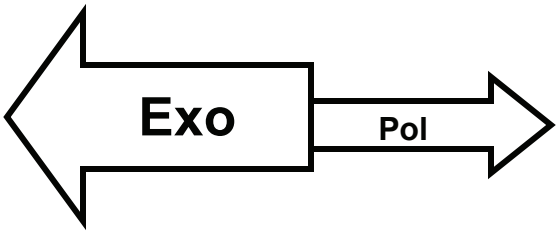
**Figure 20:** Determination of kinetics of extension of (+3) primer by exo$^-$ pol on damaged template. Visualization of products (A) and quantification of extension products (B). The reaction displayed linear kinetics throughout the time of the experiment.




**Normal Polymerization**




**Repair**




**Idling Turnover**


**Fig. 21**

**Figure 21:** Graphic representation of respective dominance of pol or exo activity in different replication contexts. Polymerization dominates under normal replication conditions with a matched primer terminus and available dNTPs. Exo activity predominates during repair of mismatched nucleotides and on ssDNA. Pol and Exo activities are effectively equal during idling turnover due to successive rounds of incorporation and excision. Idling turnover is thought to predominate at sites of AP damage.

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